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Comparison of ELISA, HPLC-FLD and HPLC-MS/MS Methods for Determination of Aflatoxin M1 in Natural Contaminated Milk Samples

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Abstract

Presence of aflatoxin M1 (AFM1) in milk should be continuously controlled in order to protect the population from risks associated with its proven toxicity and carcinogenicity. During recent years, there has been an increase in demand for development of sensitive, accurate, simple and fast method which is reliable for detection of AFM1 at low concentrations found in milk samples. For that purpose, enzyme linked immunosorbent asssay (ELISA), high performance liquid chromatography with fluorescence detector (HPLC-FLD) and high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) were optimized and validated in order to apply them for AFM1 analysis in naturally contaminated milk samples, and to assess the closeness of agreement between results of three different methods. The obtained validation parameters indicate that all three methods are suitable for determination of AFM1 in milk samples. The statistical analysis of variance between the methods and the obtained correlation coefficients indicate that there is a strong correlation between methods. All three methods are satisfactory in meeting the requirements for official control purposes.

To the best of author's knowledge, this study represents the first report of an investigation and comparison of ELISA, HPLC-FLD and HPLC-MS/MS methods for determination of AFM1 in naturally contaminated milk samples.

Keywords: Milk, aflatoxin M1, ELISA, HPLC-FLD, HPLC-MS/MS

1. Introduction

Aflatoxins (AFs) are one of the main groups of mycotoxins due to their toxicity and prevalence in food and feed. Among eighteen identified AFs,[1] aflatoxin M1 (AFM1) has demonstrated the greatest potential for presence in the human diet since it is commonly found as contaminant of milk, which is consumed daily. Furthermore, due to high intake of milk, infants and children are the population most susceptible to the effects of AFM1.[2] AFM1 is the 4-hydroxy derivative of AFB1, formed in liver and excreted into the milk in the mammary glands of lactating animals that have been fed with AFB1 contaminated diet.[3,4]

Since AFs mainly occur in tropical and subtropical regions where temperature and humidity are favorable for the growth of *Aspergillus* species and production of the toxins,[5] the greatest numbers of published studies regarding AFs occurrence are from Mediterranean and Middle East regions.[6] Even though environmental conditions in Serbia in previous decade were not favorable for *Aspergillus* growth and AFs production,[7,8] changes in weather conditions in Serbia during year 2012 had a great influence on the occurrence of AFs in maize[9,10] and AFM1 in milk samples.[11] Determined concentrations of AFM1 in milk samples was very high and risk assessment analysis showed that all age categories, especially children, are exposed with high risk related to presence of AFM1 in milk. Presence of aflatoxins in the food chain had led to the "aflatoxins crisis" in the country. During that year, there has been an increase in demand for finding of simple, accurate and fast method which is reliable for detection of AFM1 at concentrations found in milk samples.

Considering that AFM1 was included in the first group of compounds by carcinogenicity[12] and milk and its derivates are consumed daily, most countries have set maximum level (ML) of AFM1 in milk. ML of AFM1 in milk varies from 0.05 μg/kg in European Union[13] to 0.5 μg/kg established in United States.[14] Regulation for ML of AFM1 in milk in Serbia[15] was recently harmonized with European Union (EU) Regulation and adapted.[13] However, the presence of AFM1 in milk during January and February 2013 resulted in Regulation changes. During March 2013, Serbian Government changed previous ML of AFM1 from 0.05 μg/kg to 0.5 μg/kg.[16]

Since AFM1 has proven toxic effects at very low concentrations there is a need for sensitive, reliable and accurate analytical method for its determination.[17] A number of analytical methods for the determination of AFM1 are available in the literature. Numerous studies in the recent years highlighted enzyme linked immunosorbent assay (ELISA) as the most frequently used technique for that purpose, followed by high performance liquid chromatography with fluorescence detector (HPLC-FLD) and liquid chromatography-tandem mass spectrometry (HPLC-MS/MS).[18,19] Thin layer chromatography is almost abandoned as a technique since it is reliable for detection of AFM1 only for concentration above or around 0.05 μg/kg.[20] Further, recent studies have resulted in publication of a several biosensor-based methods for AFM1 determination in milk samples.[21, 22]

ELISA is defined as routine screening method which may be performed with a great number of commercially available test kits (Neogen Veratox®, Lansing, USA; Tecna S. r. l., Trieste, Italy; Ridascreen, R-Biopharm, Darmstad, Germany; Immunolab GmbH, Kassel, Germany; etc.). The major advantages of ELISA method are minimal sample clean-up and preparation, simple measurement procedure and low cost. However, the major disadvantage of ELISA method is possible cross-reactivity to similar compounds. Therefore, to avoid risk of obtaining false-positive results, confirmation by liquid chromatography based procedure is required.[23,24]

On the other hand, HPLC-FLD and HPLC-MS/MS methods for analysis of AFM1 need a clean-up process, usually using immunoaffinity columns (IAC) before detection. This step is mainly multistage, expensive and time-consuming. Furthermore, both techniques have to be operated by highly trained analysts and require the use of expensive analytical instruments.[25] In general, HPLC-FLD and HPLC-MS/MS represent the most widespread analytical techniques for quantitative purpose and also offer significant advantages over other techniques since they provide good sensitivity and detection of trace level of toxins.[26–29]

Furthermore, selection of an appropriate sample preparation and pre-concentration method are crucial steps for the isolation of AFM1 from the sample and qualitative and quantitative determination. In addition, the emphasis in the field of sample preparation during the last few decades has been focused on the minimization of solvent use, procedure steps, sample size and time of analysis.[30]

Whichever sample preparation procedure and technique for the determination of AFM1 are selected, the whole procedure must be validated according to European Decision[24] and Technical Report CEN/TR 16059:2010 from European Committee for Standardization.[31]

Despite the continuous development in the field of the mycotoxins analysis it should be noted that one of the main encountered problems is a heterogeneous distribution of mycotoxins in the sample. It is well known that total error of the mycotoxin test procedure represents the sum of sampling, sample preparation and analytical errors.[32] For that purpose, European Regulation[33] have defined protocols for sampling with the aim to provide representative sample for analysis. Those protocols are mainly confirmed for solid samples, especially cereals and nuts. However, to our knowledge, distribution of mycotoxins within the liquid samples was rarely investigated. Furthermore, there is a lack of data regarding comparison of the performance of ELISA, HPLC-FLD and HPLC-MS/MS methods for AFM1 determination. For that purpose, the aim of this study was to validate and check performance of three different methods and to apply them for AFM1 analysis in naturally contaminated milk samples.

2. Experimental

2. 1. Samples

The heat-treated skimmed (milk fat content 0.5%) and semi-skimmed (milk fat content from 0.8% to 1.2%) milk samples were analyzed in this study. Samples were collected from supermarkets in Novi Sad (Republic of Serbia) during the same day in July 2013. The examined samples were produced by two out of six largest dairy producers in Serbia. Immediately after collection, the samples were transported to the laboratory. Before analysis, the whole amount of samples in original packing material was mixed on horizontal shaker (Benchmark Scientific, Orbi Shaker, Edison, USA) in order to ensure homogeneity of the samples. After opening, the milk samples were analyzed using ELISA method and prepared for HPLC/FLD and LC-MS/MS analysis. The prepared samples were then analyzed during the same day on previously development and validated HPLC/FLD and LC-MS/MS methods.

Out of thirty milk samples analyzed using ELISA technique, fifteen were selected for further analysis, with the aim to cover three different ranges of AFM1 concentrations: < 0.1 μg/kg, 0.1–0.4 μg/kg and > 0.4 μg/kg.

2. 2. Chemicals and Reagents

Determination of AFM1 by ELISA has been done using Test kit I´screen AFLAM1 (Tecna S. r. l., Trieste, Italy). For HPLC analysis, acetonitrile, methanol and *n*-hexane (all HPLC grade, purity ≥ 99.9%) were purchased from Merck (Darmstadt, Germany). Trifluoroacetic acid (TFA, purity ≥ 99.5%) and formic acid (purity ≥ 99.9%) were obtained from Thermo Fisher Scientific (Cheshire, United Kingdom) and from Carlo Erba (Milan, Italy), respectively.

Methanol (LC-MS grade) obtained from J. T. Baker (Deventer, The Netherlands) and ammonium format (eluent additive for LC-MS, purity ≥99.0%) from Fluka Analytical (Sigma-Aldrich, Steinheim, USA) were used for HPLC-MS/MS analysis.

Ultra-pure water was produced by Milli-Q purification system (Milli-Q from Millipore, USA).

AFM1 standard with certificated concentration of 10 μg/mL was purchased from Sigma Aldrich (Prague, Czech Republic). Standard solutions were prepared in acetonitrile and stored at –10 °C. Those solutions were used for solvent based calibration (for HPLC-FLD and HPLC-MS/MS), matrix-matched calibration (for HPLC-MS/MS) and for fortification of blank milk samples (for ELISA, HPLC-FLD, HPLC-MS/MS). Standards for HPLC-MS/MS matrix-matched calibration (MMC) were prepared by adding appropriate volumes of working standard solution to blank milk samples at the final reconstitution step, over the range from the limit of quantification (LOQ) to 100 ng/mL. The standard solutions were stored under refrigerated conditions (4 °C).

Partially defatted raw lyophilized milk was used as certified reference material (CRM) (MI1142-1/CM, Progetto Trieste, Test Veritas, Padova, Italy).

2. 3. Sample Preparation and Determination of AFM1 by ELISA Method

Milk samples were prepared according to the test kit manufacturer´s instructions. Samples were centrifuged at 3000 g for 10 min (Tehtnica, Slovenia). Since all analyzed milk samples in this study were passed through homogenization process during production in dairies, centrifugation did not influence separation of phases. 100 μL of the milk was used for the analysis.

Determination of AFM1 was done by ELISA method using I 'screen AFLAM1 test kit (Tecna S. r. l., Trieste, Italy). Analyses were performed according to the test kits instructions. Procedure is based on binding of free AFM1 from samples and standard solutions to the anti-AFM1 antibodies during first incubation. Any unbound substance is removed in a washing step. A second incubation is performed with an aflatoxin-HRP conjugate, which covers all the remaining free binding sites of the antibody. The bound enzyme activity is determined by adding a fixed amount of a chromogenic substrate. The enzyme converts the colorless chromogen into a blue product during the third incubation. The addition of the stop reagent leads to a color change from blue to yellow. The absorbance is measured by a microplate reader (Thermolabsystem, Thermo, Finland) at 450 nm. The color development is inversely proportional to the AFM1 concentration in the sample. Concentration of AFM1 was calculated from calibration curve which was obtained using 7 standards with the following concentrations: 0, 5, 10, 25, 50, 100 and 250 ng/L. Samples with AFM1 concentration greater than 250 ng/L were diluted with sample diluent solution from the test kit and analyzed again.

2. 4. Sample Preparation for HPLC and HPLC-MS/MS

Fifty mL of warm milk (30–35 °C) was filtered through a Whatman No. 4 filter paper (Whatman International Ltd., Maidstone, UK) and applied to the AflaStar™ M1 R-Immunoaffinity Columns (IAC) (Romer Labs Inc., Union, MO, USA). Flow rate of the milk was approximately 1–3 mL/min. After the milk completely passed, IAC was rinsed with 20 mL of ultra-pure water. The AFM1 was eluted with 2 mL of methanol. Eluate was collected and evaporated to dryness under gentle stream of nitrogen.

Since AFM1 in milk samples occurs in low concentrations, post derivatization step for enhancing its fluorescence on HPLC-FLD is required.[34] This was achieved by adding 100 μL of TFA and 200 μL *n*-hexane to the residue from the evaporated methanol eluate or to the AFM1 working standards, vortexing for 30 s (BOECO, Germany), and keeping in the dark for 10 min at 40 °C. After evaporation, 400 μL of wather:acetonitrile (75:25, *v/v*) mixture was added to the vials and vortexed for 30 s.

For HPLC-MS/MS analysis the residue was reconstituted with 400 μL of initial mobile phase.

2. 4. 1. Determination of AFM1 by HPLC

The HPLC instrument used for determination was an Agilent 1200 (Agilent Technologies Inc., USA) system equipped with fluorescence detector (FLD), Chemstation Software (Agilent Technologies), binary pump, vacuum degasser, auto sampler and Agilent column (Eclipse XDB-C18, 1.8 μm, 4.6 × 50 mm). The mobile phase consisted of an isocratic mixture of water/acetonitrile (75:25, *v/v*) and flow rate was 0.25 mL/min. Fifteen microliters of standards and samples were injected onto the HPLC column. The fluorescence detector was set to excitation and emission wavelengths of 360 and 423 nm, respectively. The retention time of AFM1 was 3.8 min. Typical chromatograms of AFM1 standard and naturally contaminated milk sample are shown in Figure 1.

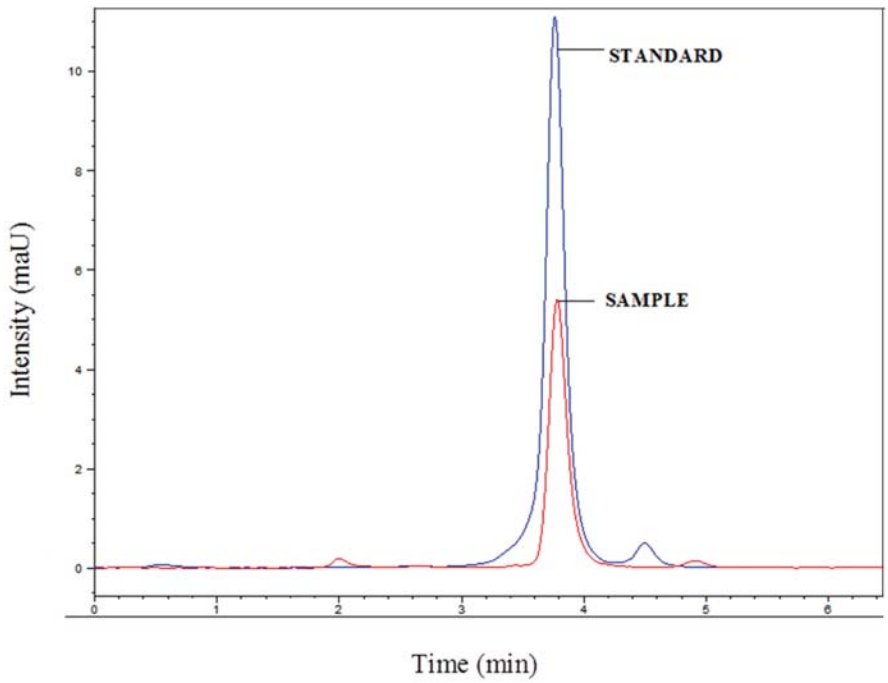

Figure 1. HPLC-FLD chromatograms of AFM1 standard (100 ng/mL) and naturally contaminated milk sample no. 14

2. 4. 2. Determination of AFM1 by HPLC-MS/MS

For HPLC-MS/MS analysis, the same HPLC system was coupled to the mass spectrometer Agilent 6410 Triple Quad LC/MS (Agilent Technologies Inc., USA). Mass spectrometer was operated with a multimode interface in positive ion mode. MassHunter workstation software version B.03.01 (Agilent Technologies Inc., USA) was used for the control of equipment, data acquisition and analysis. The following ionization conditions were used: drying gas (nitrogen) temperature 325 °C and flow rate 5 L/min, vaporizer 200 °C, nebulizer pressure 50 psi and capillary voltage 2500 V. Fragmentor voltages and collision energies were optimized during infusion of the pure standard, in the multiple reaction monitoring (MRM) mode to find precursor ion and two most intensive product ions for quantitation and qualitation. The utilized MRM transitions (precursor ion *m/z* → quantifier and qualifier *m/z*) were 329.1→273.1 and 329.1→259.1 (fragmentor 150 V, collision energy 20 eV) for AFM1.

The mobile phase consisted of eluent A containing methanol/formic acid (99:1, *v/v*) and eluent B consisting of ultra pure water/formic acid (99:1, *v/v*). Both eluents contained 5 mM ammonium formate. The linear gradient

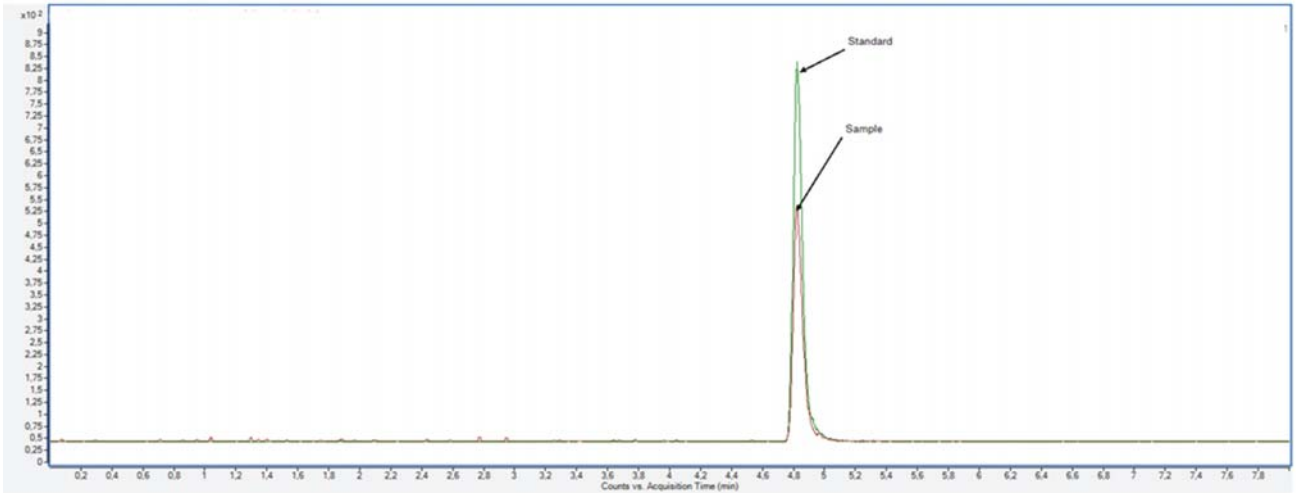

Figure 2. Extracted MRM quantifying ion (273.1) chromatograms of AFM1 standard (100 ng/mL) and naturally contaminated milk sample no. 14

program was applied from the beginning until 3.5 min with a decrease of B from 70% to 20%. Further, increase of B up to 70% was achieved in following 6 min with holdup time of 2 min. The retention time of AFM1 was 4.85 min. Typical chromatograms of AFM1 standard and naturally contaminated milk sample are shown in Figure 2.

Matrix effects were compensated by the use of matrix-matched calibration (MMC). MMC standards were prepared by adding appropriate volumes of AFM1 standard solution to blank milk samples at the final reconstitution step.

2. 5. Validation Procedure

The analytical quality of the applied analytical procedures was assured by the analysis of certified reference material (CRM) as well as spiked uncontaminated milk samples.

Partially defatted raw lyophilized milk with certified AFM1 content of 0.053 μg/kg was used as CRM (MI1142-1/CM, Progetto Trieste, Test Veritas, Padova, Italy). Uncontaminated milk samples were spiked with an appropriate amount of AFM1 standards in triplicates at three concentration levels (0.05 μg/kg, 0.5 μg/kg, 1.0 μg/kg). Those samples were left overnight in the refrigerator prior to analysis.

The validation parameters for the applied ELISA, HPLC-FLD and HPLC-MS/MS methods were determined, calculated and expressed according to Commission Decision procedure for screening and confirmatory methods,[24] as well as to Technical Report CEN/TR 16059:2010 from European Committee for Standardization.[31]

The proposed analytical procedures were validated with the respect to limit of detection (LOD), limit of quantification (LOQ), recovery, repeatability and reproducibility. Linearity was evaluated for HPLC-FLD and HPLC-MS/MS analytical procedures. Furthermore, specificity and selectivity of the HPLC-MS/MS method were characterized after analysis of matrix-matched samples. Performance characteristics of the procedures were evaluated using CRM and spiked blank milk samples.

2. 6. Statistical Analysis

Statistical analysis of variance was carried out by Duncan's multiple comparison tests using STATISTICA software version 12.[35] P values < 0.05 were regarded as significant.

3. Results and Discussion

It is evident from the Table 1 that the determined LOD and LOQ values for applied methods were significantly lower than ML established by European Union[13] (0.05 μg/kg) which indicates that proposed methods were suitable for determination of AFM1 at very low concentration.

Furthermore, LOQs for all three procedures were less than the proposed LOQ (0.02 μg/kg) for AFM1 according Technical Report CEN/TR 16059:2010.[31] According to 2002/657/EC[24] trueness of the methods were expressed after analysis of CRM. Although it is acceptable that trueness of measurements is assessed through recovery of additions of known amounts of the analyte to the unknown samples it should be pointed that the added analyte is not chemically bound in the real matrix and that therefore results obtained by this approach have lesser validity than those achieved through the use of CRM. As can be seen from the Table 1, the obtained recovery values were in accordance with recovery range (60–120%) given in Commission Decision[33] as well as in CEN/TR 16059:2010[31] (50–120%) report. Further, relative standard deviations calculated under repeatability conditions (RSD_r) using CRM analysis are shown in Table 1 and those values were in accordance with the mentioned criteria. Relative standard deviations under reproducibility conditions (RSD_R) were not determined after CRM analysis due to the small amount of available CRM.

The accuracy and precision of the methods were evaluated after analysis of spiked blank milk samples. The mean recovery (Rec %) and RSD values were determined at three concentration levels. The recovery values confirmed that the optimal recovery was obtained for all methods. Furthermore, it can be noted that ELISA method gave higher recovery values in comparison to HPLC-FLD and HPLC-MS/MS. This is in accordance with several previous studies which confirmed that ELISA gave recovery values closer to 100% in comparison to chromatographic methods.[36–38] Precision, expressed under repeatability and reproducibility conditions gave RSD_r values within the range of 6.43–8.36%, 9.53–11.3% and 11.5–9.12.8% and RSD_R values of 10.9–17.8%, 18.2–23.4% and 20.8–26.7% for ELISA, HPLC-FLD and HPLC-MS/MS method, respectively. The obtained values fulfilled the mentioned criteria of $RSD_r \leq 30\%$ and $RSD_R \leq 60\%$ (for concentration ≤ 1 μg/kg) and indicated a good precision of the methods.

Table 1. Analytical parameters for the determination of AFM1 obtained with different techniques

Technique	LOD (μg/kg)	LOQ (μg/kg)	Rec CL (μg/kg)				RSD_r CL (μg/kg)				RSD_R CL (μg/kg)		
			CRM	0.05	0.5	1.0	CRM	0.05	0.5	1.0	0.05	0.5	1.0
ELISA	0.003	0.008	107.6	94.3	95.8	96.6	3.54	8.36	7.15	6.43	17.8	14.2	10.9
HPLC/FLD	0.001	0.004	81.51	84.5	81.1	86.4	5.88	11.3	10.8	9.53	23.4	18.2	19.1
HPLC-MS/MS	0.001	0.004	71.92	74.4	72.5	80.3	8.22	12.8	11.6	11.5	26.7	21.3	20.8

Quantification of AFM1 by ELISA method was achieved using logarithmic dependence. This dependence showed good correlation (0.9908) between absorbance and AFM1 concentration. Under the optimal experimental conditions, the linearity of HPLC-FLD and HPLC-MS/MS methods was calculated from standard calibration curves (SC) in two concentration ranges: 0.5–20 ng/mL and 20–100 ng/mL. For the both methods squared correlation coefficients (R^2) were higher than 0.99, which indicated good linear correlations between AFM1 detector response and its concentration.[39] Furthermore, quantification of AFM1 by HPLC-MS/MS method requires existence of one more calibration, matrix-matched calibration (MMC). Matrix effects were calculated as signal suppression/enhancement (SSE), i.e. slope ratio for MMC and SC, which equals 90% and 95% for 0.5–20 ng/mL and 20–100 ng/mL ranges, respectively (Table 2).

Arroyo-Manzanares et al.[40] reported that values of matrix effect close to 100% indicate that there is no significant matrix effect, while values >100% and <100% indicate signal enhancement and signal suppression, respectively. Furthermore, the obtained matrix effects are in the range of ±20% which was considered as tolerable[41] and the AFM1 was quantified using matrix-matched calibration.

Since the evaluated method performance met the requirements,[31,33] those three methods were applied for analysis of naturally contaminated milk samples. At the beginning, thirty milk samples were analyzed using ELISA method and fifteen of these samples were chosen with the aim to cover three different ranges of AFM1 contamination. It should be noted that all presented results in Table 3 were corrected for recovery.

Regarding the results from Table 3, it can be seen that ELISA method did not give false positive results since presence of AFM1 determined using ELISA method were confirmed with HPLC-FLD and HPLC-MS/MS methods. Furthermore, ELISA gave slightly higher values of AFM1 concentration in comparison to HPLC-FLD and HPLC-MS/MS which is in accordance with some previously reported studies.[42,43]

The statistical analysis of variance between methods showed that significant differences were noted for the results of samples 6, 10 and 15. The obtained concentrations

Table 2. Characteristics of the calibration curves

Technique	Concentration range (ng/mL)	Solvent based calibration		Matrix-matched calibration		
		Dependence	R^2	Dependence	R^2	SSE
ELISA	0.005–0.25	y = – 0.421ln(x) + 2.5236	0.9908	–	–	–
HPLC/FLD	0.5–20	y = 1.6038x – 0.1332	0.9998	–	–	–
	20–100	y = 1.7443x + 4.0847	0.9964	–	–	–
HPLC-MS/MS	0.5–20	y = 44.762x + 11.883	0.9910	y = 40.441x + 11.301	0.9993	90.35
	20–100	y = 33.623x + 199.07	0.9982	y = 32.048 + 120.39	0.9938	94.54

Table 3. Content of AFM1 determined by ELISA, HPLC and LC-MS/MS techniques

Range of concentration (μg/kg)	Sample number	Technique ELISA MV±STD (μg/kg)	HPLC-FLD MV±STD (μg/kg)	HPLC-MS/MS MV±STD (μg/kg)
< 0.1	1	0.043 ± 0.001[a]	0.021 ± 0.017[a]	0.035 ± 0.013[a]
	2	0.036 ± 0.001[a]	0.025 ± 0.015[a]	0.029 ± 0.007[a]
	3	0.050 ± 0.001[a]	0.033 ± 0.009[a]	0.034 ± 0.010[a]
	4	0.086 ± 0.009[a]	0.074 ± 0.011[a]	0.069 ± 0.020[a]
	5	0.047 ± 0.001[a]	0.036 ± 0.013[a]	0.046 ± 0.017[a]
0.1–0.4	6	0.254 ± 0.007[b]	0.122 ± 0.028[a]	0.117 ± 0.053[a]
	7	0.231 ± 0.032[a]	0.178 ± 0.020[a]	0.180 ± 0.044[a]
	8	0.189 ± 0.013[a]	0.163 ± 0.022[a]	0.193 ± 0.020[a]
	9	0.105 ± 0.001[a]	0.075 ± 0.047[a]	0.085 ± 0.007[a]
> 0.4	10	0.253 ± 0.012[b]	0.115 ± 0.042[a]	0.112 ± 0.041[a]
	11	0.534 ± 0.070[a]	0.421 ± 0.008[a]	0.524 ± 0.033[a]
	12	0.528 ± 0.157[a]	0.356 ± 0.015[a]	0.445 ± 0.056[a]
	13	0.792 ± 0.203[a]	0.670 ± 0.075[a]	0.778 ± 0.035[a]
	14	0.440 ± 0.016[a]	0.352 ± 0.065[a]	0.326 ± 0.064[a]
	15	1.096 ± 0.226[ab]	0.760 ± 0.09[a]	1.501 ± 0.165[b]

Different letters (a, b) in the same row indicate significant differences ($p < 0.05$) between results according to the Duncan´s multiple range test
MV±STD (μg/kg): Mean value ± Standard deviation

Table 4. Correlation coefficients and correlation equations between the applied methods

Range of concentration (μg/kg)		ELISA/ HPLC-FLD	ELISA/ HPLC-MS/MS	HPLC-FLD/ HPLC-MS/MS
< 0.1	*r*	0.9748	0.9456	0.9561
	correlation eq.	y = 1.062x–0.0182	y = 0.7760x + 0.0015	y = 0.7201x + 0.0152
0.1–0.4	*r*	0.4938	0.2778	0.9572
	correlation eq.	y = 0.3258x–0.0635	y = 0.2090x + 0.0943	y = 1.0911 x–0.0052
> 0.4	*r*	0.9623	0.9851	0.9142
	correlation eq.	y = 0.6822x–0.0493	y = 1.7267x–0.456	y = 2.2602x–0.422
Whole range	*r*	0.9857	0.9651	0.9465
	correlation eq.	y = 0.7468x–0.0065	y = 1.2293x–0.0857	y = 1.5912x–0.0626

r: correlation coefficient

for samples 6 and 10 using ELISA method were significantly different in comparison to concentrations obtained using HPLC-FLD and HPLC-MS/MS methods. Further, HPLC-MS/MS analysis for sample 15 gave significantly different result in comparison with other two methods. However, despite those differences, statistical analysis indicates a good correlation between methods since in only 4 out of 45 (15 samples analyzed using 3 different methods) the obtained results were statistically different.

In order to confirm good correlation between the applied methods, simple linear regression analyses between the results of ELISA, HPLC-FLD and HPLC-MS/MS were investigated and presented in Table 4.

Correlations between methods were estimated in terms of correlation coefficients (*r*) for different ranges of concentrations. In the view of correlation coefficients between methods observed for different range of contamination it can be noted that those values were in the range from 0.2778 to 0.9748. The weak correlations, characterized with $r<0.5$, were observed between results of ELISA in comparison to HPLC-FLD and HPLC-MS/MS methods for the contamination range from 0.1 to 0.4 μg/kg. Furthermore, the obtained correlations coefficients higher than 0.9 for the whole range of quantification indicate that strong correlation exist between methods.[39] The highest correlation ($r = 0.9857$) for the whole range of quantification was observed between the results of ELISA and HPLC-FLD methods followed with correlation ($r = 0.9651$) between results of ELISA and HPLC-MS/MS. Although HPLC-FLD and HPLC-MS/MS analysis included the same sample preparation procedure (IAC) the slightly lower correlation ($r = 0.9465$) was observed between the obtained results. This fact could be explained by the possible losses of analyte during the multistage sample preparation such as applied IAC clean up procedure.[44] For that purpose Hongyo et. al,[45] suggested using the same eluted extract to increase the correlation between methods. However, Beaver et al.[46] claimed that the same eluted extract allows measuring only the effect of detection variances. Therefore different eluted extracts from the same milk sample obtained after IAC clean up procedure, were used for the further analysis on HPLC-FLD and HPLC-MS/MS in this study.

Hongyo et al.[45] reported that correlation coefficient between ELISA and others methods depends on specificity and reproducibility of the used monoclonal antibody. Further, Nilufer and Boyacioglu[47] indicated that food matrix also may have great influence on correlation between methods. Mühlemann et al.[48] confirmed this observation with obtained good correlation coefficients between methods for aflatoxins determination in peanut and oilseeds samples, and low correlation coefficients for cereals and grains.

The obtained correlations for the whole range of quantification (>0.9) in this study could indicate relatively homogeneous distribution of AFM1 in the examined milk samples although it is well known that mycotoxins are unevenly distributed in the sample. As we have previously noted, many studies confirmed heterogeneous distribution of mycotoxins mainly for solid samples[49,50] while researches for distribution of mycotoxins in liquid samples are still missing.

Furthermore, the obtained results in this study could not be completely compared to the literature data since, to our knowledge, none of the previously reported studies have focused on the comparison of ELISA, HPLC-FLD and HPLC-MS/MS techniques for AFM1 determination in naturally contaminated milk samples. Just a few studies compared results for AFM1 determination obtained by two different methods, mainly ELISA and some chromatographic method. However, our study besides investigation of three different methods contained the most detailed information in terms of method validation procedures.

Comparison of results for AFM1 determination in milk and milk products using ELISA and HPLC methods were reported in two papers.[42,43.] Colak et al. reported good correlation between ELISA and HPLC-FLD method for AFM1 determination in three types of naturally contaminated Turkish cheeses. Rodriguez Velasco et al. confirmed that AFM1 concentrations determined using ELISA procedure were higher than concentrations determined us-

ing HPLC-FLD. Furthermore, HPLC-FLD analysis showed that two out of five contaminated milk samples according ELISA were false-positives. However, an earlier investigation showed that[51] AFM1 concentration using ELISA were lower than those determined by HPLC.

In a recently published study,[52] authors compared results for AFM1 analysis using ELISA and HPLC-MS/MS methods in 250 milk samples. Samples were collected during a period of five months. Regression analysis of obtained results showed high level of agreement (R^2 = 0.920, r = 0.959). Although this study included high number of samples the data regarding type of milk samples (provenience, raw or heat treated, content of fat) were not shown. Furthermore, the data about range of AFM1 concentrations is missing. In agreement with our results, Stefanović et al.[52] reported that obtained recovery for ELISA of 110% is higher than recovery obtained by HPLC-MS/MS (between 65–81%).

4. Conclusion

This work describes the comparison of ELISA, HPLC/FLD and HPLC-MS/MS methods for determination of AFM1 in milk samples. Evaluated methods performances indicate that ELISA, HPLC-FLD and HPLC-MS/MS methods were suitable for AFM1 analysis. Furthermore, the obtained correlations coefficients for the whole range of quantification were higher than 0.9 which indicate that strong correlation exist between methods. Therefore, the obtained results for real samples verified that all three methods can be used for that purpose. Which methods will be selected depends mostly on the availability of the equipment and on the number of samples. ELISA offers many advantages including shorter analysis time, absence of complicated sample preparation steps and simplicity of the analytical procedure in comparison to time-consuming and expensive chromatographic techniques. However, a suspected or non-compliant result (>0.050 μg/kg) must be confirmed by a confirmatory method.

Due to the serious health risks associated to AFM1, the obtained results may contribute to knowledge increase in the field of analytical methods for its determination.

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Povzetek

Prisotnost aflatoksina M1 (AFM1) v mleku je potrebno stalno nadzorovati z namenom zaščite prebivalstva pred tveganji zaradi njegove potrjene strupenosti in karcinogenosti. V zadnjih letih se je pojavila večja potreba po razvoju občutljive, točne, preproste in hitre metode, ki bi zanesljivo zaznavala AFM1 v nizkih koncentracijah, prisotnih v vzorcih mleka. S tem namenom smo optimizirali in validirali metode z encimskim imunotestom (ELISA), visokoločljivostno tekočinsko kromatografijo s fluorescenčnim detektorjem (HPLC-FLD) in visokoločljivostno tekočinsko kromatografijo s tandemsko masno spektrometrijo (HPLC-MS/MS), da bi jih uporabili za analizo AFM1 v naravno onesnaženih vzorcih mleka, pa tudi da bi ovrednotili ujemanje med rezultati treh različnih metod. Pridobljeni validacijski parametri nakazujejo, da so vse tri metode primerne za določanje AFM1 v vzorcih mleka. Statistična analiza variance med metodami in pridobljeni korelacijski koeficienti nakazujejo izrazito korelacijo med metodami. Vse tri metode zadovoljivo ustrezajo zahtevam, določenim za potrebe uradnega nadzora.
Kolikor je avtorjem znano, ta študija predstavlja prvo poročilo o raziskavi in primerjavi ELISA, HPLC-FLD in HPLC-MS/MS metod za določanje AFM1 v naravno onesnaženih vzorcih mleka.