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STUDY ON THE METHOD PERFORMANCES FOR SCREENING AND CONFIRMATORY ANALYSIS OF AFLATOXIN M_1 IN RAW MILK ACCORDING TO THE MYCOTOXIN REGULATIVE REQUIREMENTS

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Screening and confirmatory methods for aflatoxin M_1 (AFM_1) analysis were optimized and studied regarding the European Decision 2002/657/EC requirements. The recoveries of AFM_1 from the spiked samples at levels between 0.025 and 0.075 μg kg^{-1} were 99.6–110.3% with precision of 10.5–13.4%, confirming the immunochemical method precision and reliability for intensive surveillance studies. According to the regulatory requirements, for confirmation of the obtained positives from the screening, the reference high-performance liquid chromatographic procedure was evaluated in reference to the performance criteria. The between-day recovery obtained was in the range 67.2–72.8%, and the precision range was 5.4–9.1%. Two hundred and twenty five positives from the screening were subjected to confirmatory analysis. Comparison of the data obtained from the immunoassay and chromatographic method (R^2 = 0.764) revealed a slight overestimation of the screening method, but no cases of false-negative determinations occurred.

Keywords: aflatoxin M_1; raw milk; immunoenzymatic assay; HPLC-FD; 657/2002/EC

ПРОУЧУВАЊЕ НА ПЕРФОРМАНСИТЕ НА СКРИНИНГ И НА ПОТВРДЕН МЕТОД ЗА АНАЛИЗА НА АФЛАТОКСИН M_1 ВО СУРОВО МЛЕКО СОГЛАСНО БАРАЊАТА НА РЕГУЛАТИВАТА ЗА МИКОТОКСИНИ

Во овој труд се оптимизирани и проучени скринингот и методот за потврдување анализа на афлатоксин M_1 (AFM_1) со оглед на барањата на Одлуката 2002/657/ЕС на Европската комисија. Од спикуваните примероци на ниво на концентрации помеѓу 0,025 и 0,075 μg kg^{-1} се добиени аналитички приноси за AFM_1 во опсег од 99,6 до 110,3 %, при што утврдената прецизност се движи во опсегот од 10,5 % до 13,4 %. На тој начин е потврдена прецизноста и сигурноста на имунохемискиот метод за негова примена при интензивни надзорни студии на голем број примероци. За релевантно потврдување на позитивните резултати од скринингот е неопходно да се изврши процена на изведбата на референтниот високо ефикасен течно-хроматографски метод, земајќи ги предвид критериумите утврдени во регулативата. Аналитичкиот принос кој е определен во текот на два дена се движи во опсегот од 67,2 до 72,8 % и прецизноста од 5,4 % до 9,1 %. Со примена на потврдниот хроматографски метод повторно беа анализирани 205 позитивни примероци од скринингот. Споредбата на податоците од резултатите добиени со скринингот и потврдниот метод (R^2 = 0,764) покажа дека концентрациите на AFM_1 кои се определени со скринингот во одредена мера се повисоки, но не се утврдени лажно негативни резултати.

Клучни зборови: афлатоксин M_1; сурово млеко; имуноензимски тест; HPLC-FD; 657/2002/ЕС

1. INTRODUCTION

Aflatoxins are secondary mold metabolites from the *Aspergillus* species. They represent a serious risk for animal and human health, due to their well-documented hepatotoxic, genotoxic, carcinogenic, teratogenic, immunosuppressive and antinutritional effects [1–2]. Aflatoxin B_1 (AFB_1) is known to be the most carcinogenic and has been classified by the International Agency for Research on Cancer (IARC) in Group 1 of human carcinogens [3]. The attention paid to the presence of aflatoxins in feed is very important because of the possible contamination of the milk produced by animals fed with aflatoxin-contaminated feed. Aflatoxin M_1 (AFM_1), the hydroxylated metabolite of AFB_1, may be found in milk and milk products obtained from livestock that have ingested contaminated feed. Generally, it is deemed that approximately 1–3% AFB_1 present in animal feed appears as AFM_1 in milk, varying from animal to animal, and depending on the time of milking, among many other factors [4]. It takes approximately 72 hours for AFM_1 to be released from the animal's body to undetectable concentrations after ingestion of feed contaminated with AFB_1. Unfortunately, AFM_1 is relatively stable towards either thermal processing or during the preparation and storage of various dairy products [5].

Since 2002, following the demonstrated toxic and carcinogenic effects of AFM_1, IARC has changed its classification from Group 2 to Group 1 [6]. For these reasons AFM_1 was included in the Group B of Annex I of Council Directive 96/23/EC [7], which comprises those substances with established maximum residue limits (MRLs). Thus, strict regulatory limits for this compound are currently in force and accurate monitoring analyses have been initiated. In the Commission Regulation [8], the maximum level of AFM_1 in raw milk, heat treated milk and milk intended for milk-based products was set at 0.050 μg kg^{-1}, while the MRL for infant formulae was set at 0.025 μg kg^{-1}.

A high performance liquid chromatography analysis with fluorometric detection (HPLC-FD) combined with immunoaffinity clean-up procedure is the basis of the standard method designed and used for the determination of AFM_1 in milk [9]. Beside this, in the past decade, several methods based on the technique mentioned above have been reported [10–12]. HPLC-FD is mostly used for the determination of AFM_1 because of the characteristics of specificity, high sensitivity and relative simplicity of operation. However, the main disadvantage of this technique is a low throughput due to the time-consuming clean-up procedure, making it inadequate for intensive surveillance studies.

An LC/MS/MS methodology, either employing high performance or ultrahigh performance chromatography, has been shown to offer significant benefits for AFM_1 analysis, as it provides good sensitivity and confirmation of the analyte. Several papers describing liquid chromatography–tandem mass spectrometric for determining AFM_1 in different kinds of foodstuffs have been published [13–16]. However, despite the favorable characteristics of this technique for AFM_1 confirmation in milk and milk products, the main, and in most of the cases, crucial disadvantage is the availability, lower cost-effectiveness benefit comparably to HPLC-FD, and the need for highly trained personnel.

In the past, several procedures for the determination of AFM_1 have been developed, comprising immunochemical methods [17–22], such as enzyme-linked immunosorbent assays (ELISA), which are commonly used for screening purposes within intensive surveillance programs. The method should be rapid, simple, with less sample clean-up procedures, high throughput and performances that comply with the requirements laid down in the ISO Standard [9].

In fact, each method intended for performing official food controls for mycotoxins shall be validated not only in a collaborative trial study but also by following the validation guidelines aimed at the attainment of minimum performance criteria, as defined in the Commission Regulation 401/2006/EC [24]. Validation procedures of analytical methods are necessary in order to provide accurate and reproducible results within- and inter-official control laboratories involved in the monitoring and risk-assessment studies.

The Commission Decision 2002/657/EC [25] was adopted with purpose of ensuring both the quality of methods and the correct interpretation of the analytical results attained by official control laboratories. Nevertheless, Regulation 401/2006/EC [24] establishes performance criteria for methods of analysis of AFM_1 in milk, but does not describe the validation procedures that should be undertaken. Therefore, it would be convenient to follow the validation approach of Decision 657/2002/EC [25], thus verifying the minimum criteria described in the specific mycotoxin regulation [24].

In spite of the many published articles for ELISA screening for the determination of AFM_1 in milk samples, only a few comprehensive validation studies have been reported [18, 19, 21, 22], which still do not strictly follow the Decision 2002/657/EC

[25] and ISO Standard requirements [9]. In this paper, an in-house evaluation of the performance characteristics of a commercial ELISA kit for the rapid screening of AFM_1 in raw milk samples following the related regulative [9, 24, 25] was performed. The method was validated using the conventional approach of the Commission Decision [25], and the procedures for determining selectivity, recovery, precision, and detection capability were reported. Applying the validated method, a number of raw milk samples were analyzed within the surveillance program conducted during 2013. It is strictly required for the positives obtained from the screening testing to be confirmed by the employment of chromatographic techniques coupled to a spectrometric detection system, either fluorescence or mass spectrometric [25]. For that purpose, a confirmatory HPLC-FD method, based on the ISO reference method [23], has been optimized and validated, applying again the Decision 2002/657/EC approach [25], but on this occasion for confirmatory methods. Furthermore, the methods were tested for their validity through participation in FAPAS proficiency testing in spiked sample material – milk powder. The determined concentrations of AFM_1 determined by ELISA and HPLC-FD method were compared, and the correlation obtained by applying the regression analysis was evaluated.

2. EXPERIMENTAL

2.1. *Chemicals and standard solutions*

Acetonitrile and water were of HPLC grade and were purchased from Carlo Erba (Milano, Italy). The Trilogy reference standard of AFM_1 with a concentration of 0.5 μg ml^{-1}, was purchased from R-Biopharm (Darmstadt, Germany). The solution was kept at +4 ºC until the date of expiry declared by the manufacturer. An intermediate standard with a concentration of 10 ng ml^{-1} was prepared in 10% acetonitrile solution and kept at –20 ºC for one month. For construction of the calibration graph, a series of standards with concentrations 0.075 ng ml^{-1}, 0.125 ng ml^{-1}, 0.25 ng ml^{-1}, 0.50 ng ml^{-1}, 1.25 ng ml^{-1}, 2.50 ng ml^{-1}, 5.0 ng ml^{-1} and 10 ng ml^{-1}, was prepared by suitable dilution in 10% acetonitrile, weekly, and were kept at –20 ºC. The stability of the solutions was not the subject of interest in this study; therefore, the previously published recommendations were followed [12].

2.2. *ELISA screening method*

ELISA kit Immunoscreen AFM_1 (Tecna, s.r.l., Trieste, Italy) was strictly used according to the manufacturer's instructions. After cold centrifugation at +4 ºC, samples were analyzed in duplicate, on the 96-well plate which had been coated with anti-AFM_1 antibodies. Free AFM_1 molecules were bound to the anti-AFM_1 antibodies, and the unbound sites were covered with aflatoxin-HRP conjugate. The bound enzyme activity after colorization with the appropriate chromogen was determined using a microplate photometer Bio-Rad Model 680 (Philadelphia, USA), set at 450 nm. AFM_1 levels in samples were inversely calculated with the use of a six-level calibration curve, within the concentration range 0.005–0.250 ng ml^{-1}, and a zero standard.

2.3. *HPLC analysis*

Positive ELISA samples, *i.e.* all samples with AFM_1 either above the MRL, or above the legally acceptable level (detection capability of the screening method) were analyzed with the confirmatory HPLC-FD method, based on the ISO standard method [23]. The overall analysis comprised two steps: AFM_1 extraction from the milk samples and HPLC-FD analysis of the extract. In particular, extraction and purification were performed with AFLA M_1 HPLC immunoaffinity columns (VICAM, Milford, MA, USA). When passing 25 ml of milk sample, AFM_1 was initially retained by the monoclonal antibodies' gel suspension, and afterwards eluted with 2 ml acetonitrile that had been passed twice through the column. Then, the eluate was evaporated under a stream of nitrogen at 35 ºC, nearly to dryness, and made up to 0.5 ml with 10% acetonitrile in water. The extract was submitted to HPLC analysis, performed on an Alliance system (Waters, Milford, MA, USA) consisting of a 2695 separation module and a 2475 multi-wavelength fluorescence detector. Separation of AFM_1 was performed isocratically, at ambient temperature, on a C_{18} end-capped analytical column (MERCK, Darmstadt, Germany). As a mobile phase, acetonitrile:water mixture (25:75, v/v) at 1 ml/min flow rate was applied. The fluorescence detector was set at 365 nm for the excitation, and 435 nm for the emission wavelength.

2.4. *Validation of the screening method*

Performance characteristics of immunochemical methods were determined as prescribed for qualitative screening methods in Commission Decision 2002/657/EC [25]. The limit of detection (LOD) and limit of quantitation (LOQ) were determined by measuring the signal of thirty repetitive blank samples, in one batch, which consisted

of fifteen bovine and fifteen ovine raw milk samples. From the calibration curve, a half maximal inhibitory concentration (IC_{50}) was determined as a measure for AFM_1 inhibition effectiveness of the specific antibodies. Recovery was calculated from the repeated measurements of ten fortified blank sample replicates, at ½ MRL, MRL and 1½ MRL. From the recovery experiments, the method precision, expressed with the coefficient of variation (CV) was also obtained. CCβ was determined by fortifying 15 bovine and 15 ovine raw milk samples at a level lower than the MRL. The calculations for the detection capability (CCβ) were performed by the formula provided in the EU Commission Decision [21]. Specificity of the assay towards AFM_1 and potential cross-reactants was previously described elsewhere [20, 26].

2.5. *Validation of the confirmatory method*

The optimized HPLC-FD method was validated according to the validation protocol foreseen in the Decision 2002/657/EC [25]. LOD and LOQ were evaluated from the slope (b) of the matrix calibration curves and the residual standard error ($s_{y/x}$) by means of following equations LOD = 3.3 $s_{y/x}/b$; LOQ = 10$s_{y/x}/b$ [27]. Recovery and precision were determined by spiking tested blank milk material by HPLC-FD, at ½ MRL, MRL and 1½ MRL level in six replicates on two different days. Additionally, trueness was estimated by testing certified reference material (CRM) MI11-I/CM lyophilized milk (Progetto Trieste, Italy). Decision limits (CCα) and detection capability (CCβ) were determined and calculated according to the procedure and formula provided in the Decision [25]. Validation data, together with a bottom-up approach, were used for the evaluation of measurement uncertainty, as suggested by the EURACHEM/CITAC Guide [28]. Hence, uncertainties can be listed as class A, due to casual errors attained for replicate measurements, and class B derived from external sources such as instrumental calibrations, reference materials and standard purity. Following the published authors suggestions [12, 29] the uncertainty components were identified, expressed as RSD values, and classified in groups A and B (Table 3). On the basis of uncertainties propagation law, the AFM_1 relative uncertainty was calculated by the proposed equation [12] written in a general form as:

$$\bar{u}\left(c_{AFM_1}\right) = \sqrt{\sum u_i^2} \qquad (1)$$

where $\bar{u}$ indicates the relative uncertainty of measurement, c_{AFM_1} is the analyte concentration in the sample, and u_i^2 is the uncertainty of each component listed in Table 3. The calculated relative measurement uncertainty was converted to expanded measurement uncertainty using a coverage factor k = 2, corresponding to a 95% confidence level [28].

Furthermore, laboratory performances were evaluated by taking part in a FAPAS (Food and Environmental Research Agency, York, UK) 04217 proficiency testing round in matrix – milk powder.

2.6. *Milk sample materials*

Bovine and ovine milk samples were previously tested by HPLC-FD to confirm that they were blanks. Samples with AFM_1 content below the detection limit of the method applied were considered to be free of AFM_1. These samples were used for spiking purposes during validation of the screening method.

In total, 3407 raw milk samples were tested for the presence of AFM_1 applying the screening method, in the period February–December 2013. Samples containing AFM_1 higher than the detection capability value of the screening method, estimated through the validation procedure, were subjected to confirmatory analysis by the HPLC-FD method. Two hundred and five milk samples were considered to be possibly non-compliant and tested further for final judgment of the sample compliance. Since the stability of AFM_1 in real samples is well known, a stability study was not performed, and samples were kept at +4 ºC for 48 hours, or at –20 ºC, following recommendations by other authors [30].

3. RESULTS AND DISCUSSION

3.1. *Validation of the ELISA method*

3.1.1. *Calibration curves*

The ELISA curve for AFM_1 in assay buffer was logarithmic, and the responses of the concentrations are expressed as B/B_0 ratio in % (Fig. 1), where B is the absorbance at a given concentration of the analyte and B_0 the absorbance of the zero standard. The stability and repeatability of the AFM_1 calibration curves was assessed from ten replications obtained over a three month period. The CVs calculated for individual calibration points of the AFM_1 standard curves ranged from 3.48% to 12.17%. The main IC_{50} value (corresponding to 50% binding inhibition) for the ten repeated assays was 0.029 ± 0.005 μg kg^{-1} (Table 1). IC_{50} value is a

quantitative characteristic for the assay sensitivity – lower IC_{50} indicates higher sensitivity. The obtained sensitivity in this study was lower than 0.050 μg kg^{-1}; thus, this is in compliance with the recommendations of the ISO Standard [9].

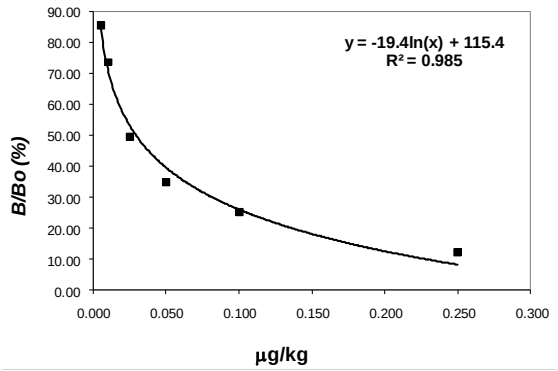

Fig. 1. Calibration curve for AFM_1 in the range 0.005 μg kg^{-1}–250 μg kg^{-1} (N=10)

3.1.2. *Limit of detection, limit of quantification and detection capability*

Following the procedure explained in the experimental section, the obtained data for LOD and CCβ are presented in Table 1. The calculated LOD of 0.0066μg kg^{-1} (Table 1) was significantly lower than the MRL value for AFM_1 (0.050 μg kg^{-1}), thus avoiding the false negative results, and obtaining a reasonable number of false positives. The obtained values were higher than those published by other authors [19, 30, 31]. This was expected, bearing in mind the fact that other authors have optimized the ELISA method by performing the experiments only on bovine milk. In this study, a robustness factors arising from the different chemical compositions of bovine and ovine milk samples were taken into account, and their influence on method precision and sensitivity is obvious. This approach is convenient for the validation of screening methods for residues of veterinary drugs [32], and with negligible negative impacts on the LOD and precision, it was successfully applied for screening of AFM_1.

The screening procedures do not need to be fully quantitative, but they should unambiguously indicate the presence of the target analyte at regulatory levels [25]. For this reason, the detection capability (CCβ) of the assay was assessed (Table 1) with respect to the established MRL for AFM_1 [8]. Since this parameter is defined as the lowest concentration that can be determined with an error probability of β (5%), it should avoid the problem of false negatives. The obtained value for CCβ was lower than the established MRL for AFM_1 [8], which is in agreement with the Decision 2002/657/EC [25] requirements.

3.1.3. *Assessment of the recovery and precision*

To ascertain the information of matrix interferences on the assay precision and accuracy, a set of blank samples, both from bovine and ovine milk were fortified with AFM_1 at concentrations of 0.025 μg kg^{-1}, 0.050 μg kg^{-1}, and 0.075 μg kg^{-1}, and carried through the ELISA procedure. The results from these experiments are presented in Table 1. Mean recovery values were calculated from ten individual measurements at each concentration level, applied in duplicate on the microtiter plate (N = 20). The signals from the blanks were not subtracted from the signals of the fortified samples; thus, higher recovery values were revealed in comparison to some other authors [30], and very similar to authors using the same manufacturer's ELISA kit [31]. The same is valid for the RSD values as well; they were somewhat higher, and reflected the variances in bovine and ovine milk samples. Accordingly, the recovery rate and precision were fully in line with the recommendations laid down in Decision 2002/657/EC [25], regarding the screening methods.

T a b l e 1

Validation parameters for the ELISA screening method for AFM_1 in raw milk

Validation parameter	**Obtained value**
Limit of detection (μg kg^{-1})	0.0066
Limit of quantification (μg kg^{-1})	0.022
$IC_{50} \pm SD$ (μg kg^{-1})	0.029±0.005
Detection capability (μg kg^{-1})	0.047
Recovery at 0.025 (μg kg^{-1}/%)	99.6 (10.5)*
Recovery at 0.050 (μg kg^{-1}/%)	110.3 (11.9)*
Recovery at 0.075 (μg kg^{-1}/%)	105.3 (13.4)*

*RSD expressed as %

3.1.4. *Test quality control in routine analysis*

During the test application, quality control (QC) procedures were established for monitoring the test validity. The internal QC strategy was based on the Shewhart control charts approach [33]; hence, the method stability was tested over a six month period. As a control material, blank sample fortified with AFM_1 at the MRL level was employed. It was confirmed (Fig. 2) that the recovery was stable over the tested period of time, so the test results were considered stable, too.

Additionally, except for the internal QC, the method validity was tested by participation in FAPAS 04217 AFM_1 proficiency testing round (May–June 2013), with spiked milk powder as a test material. The obtained *Z*-score for the ELISA test was –0.2, confirming the high method precision and trueness.

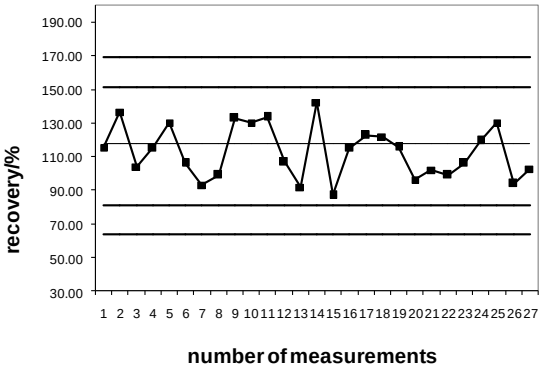

Fig. 2. Shewhart control chart for the recovery: (a) mean recovery, (b) lower action limit (mean – 3SD), (c) lower warning limit (mean – 2SD), (d) upper warning limit (mean + 2SD) and (e) upper action limit (mean + 3SD). The spiking level: 0.050 μg kg^{-1}

3.2. *Validation of the HPLC-FD method*

With the purpose of evaluating the in-house performances of the confirmatory HPLC-FD method for AFM_1 in raw milk samples, a comprehensive method validation according to the EU regulations requirements [24, 25] was undertaken. Due to the fact that no MRL exceeding was observed for ovine milk samples, the validation was limited only to bovine milk. This was not unexpected, due to the different ovine diet, which is mainly consisted of fresh and barley-based composition feed.

3.2.1.*Method selectivity towards interferences*

Selectivity seems to be one of the main problems needing to be solved in order to perform accurate analysis. Some publications [34, 35] suggest that the HPLC-FD method exhibits low selectivity as a reference method for AFM_1 analysis in raw milk samples. On the basis of Decision 2002/657/EC [25], selectivity has to be performed before proceeding with the validation approach, with the aim of confirming the method's ability to discriminate between the analyte and closely related substances or matrix interferences. A further problem presented by this approach is the carryover of impurities between runs, which was overcome by running small batches of samples (not more than ten), and reagent blank samples within the batch. As can be seen from Figure 3, which shows typical chromatograms obtained for blank, standard and spiked milk samples, respectively, the confirmatory method is able to distinguish between the analyte and other matrix components, i.e. no matrix interfering substances at the retention time of AFM_1 – 8.890 min ± 2.5% were observed.

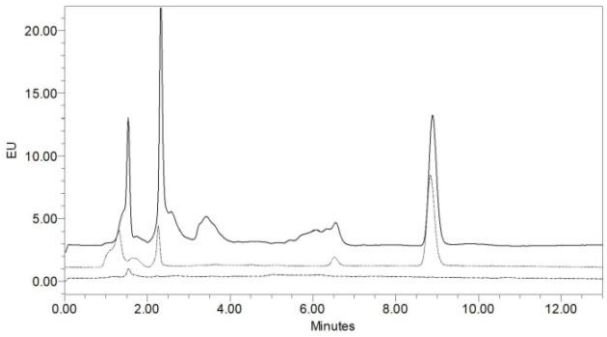

Fig. 3. Typical chromatograms obtained by the proposed method for blank milk samples, standard and spiked milk samples with AFM_1 at 0.050 μg kg^{-1} (from bottom to top).

3.2.2. *Calibration curves and limits of detection and quantitation*

Method linearity was determined by triplicate injections of eight standard solutions of AFM_1 in solvent, with concentrations of 0.075, 0.125, 0.25, 0.5, 1.25, 2.5, 5.0 and 10.0 ng/ml, corresponding to 3.75, 6.25, 12.5, 25.0, 62.5, 125, 250 and 500 ng kg^{-1} in the matrix. The calibration curve in solvent, obtained by plotting the peak area of the three replicates, and expressed in luminescence signal units versus AFM_1 concentration in the defined range, gave the linear regression equation $y = 872982x - 60049$, with a correlation coefficient (R^2) of 0.9996. The corresponding calibration curve in the matrix revealed a regression equation $y = 69586x - 42399$ with R^2 of 0.9991. The slope and intercept of both calibration curves were statistically compared by the *t*-Student test at a 95% confidence level. Comparisons showed no difference between calibration curves obtained from solvent solutions and spiked blank samples. Therefore, for routine analysis, calibration curves from standards in solution were used.

LOD and LOQ values were 0.05 μg l^{-1} and 0.15 μg l^{-1}, respectively, corresponding to 0.001 μg kg^{-1} and 0.003 μg kg^{-1} of AFM_1 in milk samples, calculated from the slope and residual standard error of the matrix matched calibration curve. LOQ is satisfactory with respect to MRL of 0.050 μg kg^{-1} for raw milk, heat-treated milk and milk intended for milk-based products.

3.2.3. *Precision and recovery*

Precision and method recovery were determined according to Decision 2002/657/EC [25], by performing tests on two sets of blank raw milk samples (six replicates each) fortified with AFM_1 at concentrations of 0.5-, 1.0- and 1.5-times the MRL. Samples were analyzed on two different days with the same instrument and the same operator, corresponding to a total number of 36 samples. Precision and recovery results are reported in Tables 2 and 3, respectively.

T a b l e 2

Repeatability and within-laboratory reproducibility for the determination of AFM_1 in spiked blank raw milk samples

Fortified concentration (μg kg^{-1})	Estimated parameter	Day 1*	Day 2*	Overall**
0.025 (0.5 MRL)	Mean (μg kg^{-1})	0.017	0.019	0.018
	Mean recovery (%)	69.0	76.7	72.8
	SD (μg kg^{-1})	0.001	0.002	0.002
	RSD (%)	7.4	7.7	9.1
0.050 (1.0 MRL)	Mean (μg kg^{-1})	0.032	0.035	0.034
	Mean recovery (%)	64.6	69.7	67.2
	SD (μg kg^{-1})	0.002	0.003	0.002
	RSD (%)	6.3	7.6	6.8
0.075 (1.5 MRL)	Mean (μg kg^{-1})	0.052	0.055	0.053
	Mean recovery (%)	71.2	72.2	71.7
	SD (μg kg^{-1})	0.003	0.002	0.003
	RSD (%)	6.0	4.6	5.4

*Six replicates at each level
**Twelve replicates at each level

T a b l e 3

Classification of the measurement uncertainty components and relevant values

Class of uncertainty	Description of the component	Uncertainty value (RSD/%)
A	Intra laboratory reproducibility	6.8
	Calibration curve uncertainty	1.8
	Method recovery uncertainty	4.5
B	Volume uncertainty	0.2
	Mass uncertainty	negligible
	Analytical standard uncertainty	1.0

According to Regulation 401/2006/EC [24], the maximal permitted value of the estimated experimental RSD for each concentration level has to be lower than twice the value obtained by Horwitz equation [25], independent of the matrix and analytical method used. For mass fractions lower than 120 μg kg^{-1}, some authors suggest that RSD can be assessed in a better manner by the Thompson equation [12, 36], which provides a value of 44% as the maximum permitted inter-laboratory reproducibility and 29% as the maximum permitted repeatability. As can be seen from the data presented in Table 2, both precision parameters were lower than those calculated by the Thompson equation, indicating that the method satisfies the minimum performance criteria required by the respective mycotoxin regulative [24].

The recoveries determined on two different days at the respective concentration levels presented in Table 2 were in the range of 64.6–76.7%, with repeatabilities in the range of 4.4–7.7%. The inter-day and inter-level mean recovery value, which was used to correct routine analysis results, was 71.4%. The recovery values achieved in this study were lower than those reported by other authors [11, 12, 19]. However, they are still in agreement with the performance criteria defined in 401/2006/EC [24], which recommends values in the range of 60–120% for concentrations in the range 0.010–0.050 μg kg^{-1}, and 70–110% for concentrations above 0.050 μg kg^{-1}. The lower recovery rates obtained may be due to different experimental conditions from that defined in the standard method [23], where it was required to carry out the procedure with daylight excluded as much as possible. The short-term stability of the test results for AFM_1 was assessed by the determination of recoveries on two different days. From overall recoveries and respective RSD, it can

be noticed that the method revealed stability over the tested period of time.

Additionally, the method trueness was tested on CRM-spiked milk powder with an assigned value for AFM_1 of 52.9 ng kg^{-1} and an interval of confidence 26.5–79.4 ng kg^{-1}. The determined concentration was 47.3 ng kg^{-1}, and trueness obtained was –5.6 ng kg^{-1}.

3.2.4. *Decision limit, detection capability and expanded measurement uncertainty*

The terms for decision limit (CCα) and detection capabilities (CCβ) were originally introduced in Decision 657/2002/EC [25], with the aim of assisting analytical food scientists with the interpretation of results regarding the established regulatory limits. In the case of substances with MRLs, the CCα and CCβ of a confirmatory method must be greater than the MRL and α and β errors must be equal or less than 5%. CCα represents an index of results dispersion around the MRL value, and as underlined by the mycotoxin regulative [24], this parameter is a useful tool for evaluating the uncertainty of measurements without applying the EURACHEM/CITAC approach [28]. Applying the formulas proposed by Commission Decision 2002/657/EC [25] and other authors [12], the obtained values were 0.057μg kg^{-1} and 0.064 μg kg^{-1} for CCα and CCβ, respectively.

The method uncertainty was evaluated by applying the bottom-up method suggested by EURACHEM/CITAC guide [28], using the validation data and quality assurance results. Accordingly, the calculated expanded measurement uncertainty for concentration of AFM_1 in samples around the MRL value (0.050 μg kg^{-1}) was estimated to be 0.008 μg kg^{-1} (16.8 %). As reported in Regulation 401/2006/EC [24] for food of animal origin, and in accordance with Decision 2002/654/EC [25], the measurement uncertainty can also be calculated as the difference between the CCα and MRL values. The estimated uncertainty by this approach was 0.007 μg kg^{-1} (14.5 %), yielding results that were very close to the expanded relative uncertainty calculated with the EURACHEM/CITAC guide [28].

3.2.5. *Quality control of testing*

As in the case of the screening method, besides the internal quality control applying Shewhart control charts [33] (results not shown), the method trueness was tested by participation in FAPAS 04217 aflatoxin M_1 proficiency testing round (May–June 2013), with spiked milk powder as the test material. The obtained *Z*-score for the HPLC-FD test was –0.9 confirming the high method precision and trueness.

3.1.6.Comparison of ELISA and HPLC performance for testing milk samples

In total, 3407 raw milk samples were tested from February until December 2013 for the presence of AFM_1 by applying the screening method. Samples containing AFM_1 higher than the CCβ value of the screening method, which was estimated to be 0.047 μg kg^{-1}, were subjected to confirmatory analysis by the HPLC-FD method. Two hundred and five milk samples (6.02%) were considered to be possibly non-compliant and tested further for the final determination of sample compliance. A total of 140 samples (68.3%) were confirmed to contain AFM_1 over the established MRL value. Predominantly, the false positives with ELISA were in the concentration range from 0.047 μg kg^{-1} to 0.100 μg kg^{-1}. In cases where the ELISA values obtained were over the upper range of the test, i.e. over 0.250 μg kg^{-1}, the HPLC method revealed higher AFM_1 concentrations.

The levels of AFM_1 determined by ELISA are correlated with data obtained by HPLC (Fig. 4). The graph is based on the ELISA results obtained in the range 0.047–0.250 μg kg^{-1}. In this case, the linear regression equation representing the correlation between the results obtained by the two methods was $y = 0.987x - 0.017$, with $R^2 = 0.764$ ($P < 0.01$), showing a certain overestimation of AFM_1 content by ELISA. To some extent, this overestimation explains the rate of false positives obtained by this method.

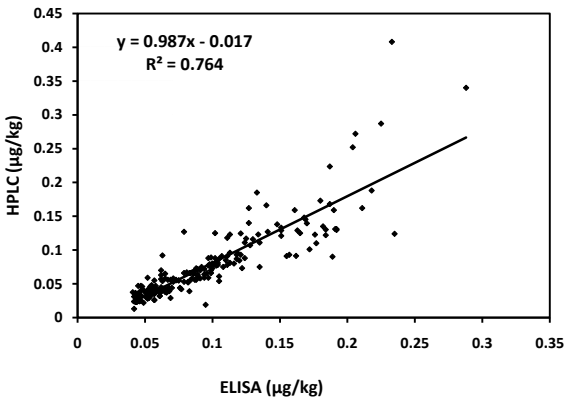

Fig. 4. Correlation between the ELISA and HPLC methods for the AFM_1 analysis in milk samples ($N = 205$)

Nevertheless, a certain overestimation of the ELISA results was observed in other similar studies [19, 37], when naturally contaminated samples were tested, independently from the type of milk samples. This was explained to be due to the higher non-specific interaction between the test antibodies and substances with similar properties as the tested analyte. Other authors [26] obtained a significant correlation ($R^2 = 0.978$) between the levels of AFM_1 determined by ELISA and HPLC.

4. CONCLUSION

The validation data for the screening immunochemical method obtained in this study, according to the Decision 2002/657/EC [25] requirements, confirm that ELISA can be considered a reliable analytical method to discriminate between compliant and non-compliant raw milk samples regarding the MRL level. The application of this assay provides an accurate, sensitive and high-throughput screening method for all types of raw milk samples in a cost-effective way. The technical competence of the method was confirmed through successful participation in the proficiency testing round. The samples presumed to be positive must be confirmed by a reference method according to the regulatory requirements. An optimized analytical method based on immunoaffinity column clean-up followed by HPLC-FD analysis was validated following the respective regulative recommendations [25]. The results of the validation process demonstrated the agreement of HPLC-FD method performances (recovery and within-laboratory reproducibility) with the Regulation 401/2006/EC provisions [24]. The estimated expanded measurement uncertainty, and excellent results achieved in a proficiency testing round confirmed the method fitness for the determination of AFM_1 in milk samples. Correlation of the two data result sets for AFM_1 obtained from the ELISA and HPLC-FD method exhibits good agreement ($R^2 = 0.764$) with a certain indication of a slight overestimation of the immunochemical assay results, affected by the so-called matrix effect.

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