Talanta 130 (2014) 8–19

Contents lists available at ScienceDirect

Talanta

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

Determination of mycotoxins in feedstuffs and ruminant's milk using an easy and simple LC–MS/MS multiresidue method

E. Tsiplakou [a], C. Anagnostopoulos [b,*], K. Liapis [b], S.A. Haroutounian [c], G. Zervas [a]

[a] Department of Nutritional Physiology and Feeding, Agricultural University of Athens, Iera Odos 75, GR-11855 Athens, Greece
[b] Pesticide Residues Laboratory, Department of Pesticides Control and Phytopharmacy, Benaki Phytopathological Institute, GR-145 61 Kifissia, Greece
[c] Laboratory of General Chemistry, Science Department, Agricultural University of Athens, Iera Odos 75, GR-118 55 Athens, Greece

ARTICLE INFO

Article history:
Received 28 January 2014
Received in revised form
5 June 2014
Accepted 11 June 2014
Available online 30 June 2014

Keywords:
Animal feed
LC–MS/MS
Milk
Mycotoxins
Cows
Sheep
Goats

ABSTRACT

Mycotoxin contamination is a common problem on feedstuffs, that can be formed on crops in the field, during harvest, storage, processing or feeding. The scope of the current study was to investigate the levels of Aflatoxin B_1, Aflatoxin B_2, Aflatoxin G_1, Aflatoxin G_2, Diacetoxyscirpenol, Ochratoxin A, Toxin HT-2, Toxin T-2 and zearalenone in a variety of feedstuffs (maize silage, alfalfa hay, cottonseed cake, corn grain and concentrates) fed to ruminants and the possible contamination of milk though consumption. For this purpose an easy and simple multiresidue LC–MS/MS method without any clean-up step was developed and successfully validated in feed and milk matrices. The LOQ of the method was set at 10 μg/kg for all analytes and 0.05 μg/kg for Aflatoxin M1 and Ochratoxin A in milk. The results showed that 7 cottonseed cake samples, out of 13 were contaminated with Aflatoxin B_1 at a level higher than the maximum levels as set by EU Regulations and with Toxin T-2 with values ranging from 8 to 562 μg/kg. Nine maize silages and 6 alfalfa hay samples were contaminated with Aflatoxin G_2 at levels higher than the maximum tolerance limit. No mycotoxins or their metabolites were found above the LOQ in any of the analyzed milk samples.

1. Introduction

Mycotoxins are low molecular weight secondary metabolites produced mainly by fungi belonging to the genera *Alternaria*, *Fusarium*, *Aspergillus* and *Penicillium* [1]. Mycotoxin production may occur in the field, during harvesting, transportation or storage and under favorable conditions of temperature, humidity, sufficient oxygen and presence of the fungal spores [2].

The most common classes that occur in feedstuffs include aflatoxins, Ochratoxin A (OTA) and trichothecenes [3]. Aflatoxins are produced by *Aspergillus* species, a family which includes Aflatoxin B_1, B_2, G_1 and G_2. Aflatoxin B_1 (AFB_1), is usually found in the highest concentration in animal feedstuffs [4]. OTA is produced by *Aspergillus* and *Penicillium* species and is a complex compound consisting of OTα linked through a 7-carboxy group to L-B phenylalanine by an amide bond [5]. Trichothecenes are produced by *Fusarium* species and constitute a large group of mycotoxins. One of the most prevalent mycotoxin of this group is T-2 toxin [6].

A mycotoxin contaminated diet may induce to the animals' acute toxicity resulting of a high level dose or chronic, resulting of long term exposure and low level dose. The animals which consume feedstuffs contaminated with mycotoxins have direct consequences to reduced feed intake, production, reproduction, weight gain and feed efficacy. Further that mycotoxins in feedstuffs can cause to the animals carcinogenesis, teratogenesis and immune system suppression as a result of chronic toxicity even at low levels [7,8].

The problem with mycotoxins does not just end in animals. Mycotoxins present in animal's feeds can be transferred to animal products mainly in milk and can pose a threat to human health [9]. Considering both the carry-over into milk and the associated risk for consumers of milk products and the adverse effects on animal health, the European Commission (EC) has established regulations for aflatoxins and OTA, but not for T-2 toxin and OTα in animal feeds [9].

Many studies with controlled feeding experiments have examined the presence of individual mycotoxins residues or their metabolites in ruminant's milk using high intake levels, usually higher than the maximum levels set by the European Commission (EC) [10]. Further to that, the fact that ruminant diets have variable composition consisted from forages, concentrates, agroindustrial by-products and preserved feedstuffs (silage) has as a result the risk of exposure to more than one mycotoxins [11]. For the above reasons more knowledge is required on the transferred mycotoxins to milk, even at low levels, to develop better risk management strategies.

* Corresponding author. Tel.: +30 2108180364.
E-mail address: c.anagnostopoulos@bpi.gr (C. Anagnostopoulos).

http://dx.doi.org/10.1016/j.talanta.2014.06.018
0039-9140/

Gas (GC) [2] and liquid (LC) chromatographies [12] are most commonly used analytical methods for the determination of mycotoxins in food and feedstuffs. Methods based on GC require a derivatization step in order to perform the analysis. Due to that, LC combined with mass spectrometry (MS) has become an important tool for the analysis of various mycotoxins in different feed matrices [12]. The combination of LC with tandem mass spectrometry (MS/MS) is a very powerful tool that provides high selectivity in the determination of multiple chemical classes of mycotoxins even in samples with minimum sample preparation steps. Today, samples are analyzed not only for mycotoxins but for several other compounds as well, like pesticide residues. Therefore the obvious next step is to try to minimize analytical work and to combine analytical methods capable to extract or even determine contaminants of different origins. Analytical methods with such a wide scope, including mycotoxins, are scarce [13].

In the current study the contamination of feedstuffs and milk from dairy ruminants fed by them, under practical farming conditions, was investigated. The scope of the method includes several of the most important mycotoxin categories such as aflatoxins, ochratoxins, trichothecenes and most widely found compounds like ZON. For the determination of the mycotoxins a fast and easy LC–MS/MS analytical method without a cleanup step, for the simultaneous determination of several mycotoxins and metabolites in animal feed matrices and milk, was developed and validated.

2. Materials and methods

2.1. Experimental design and samples collection

A total of 151 different feed samples were collected. The sampling was performed on January 2013. The samples consisted of maize silage ($n=15$), alfalfa hay ($n=42$), cottonseed cake ($n=13$), corn grain ($n=16$) and concentrates ($n=65$). A "bulk sample," composed from several primary samples, was randomly taken from the whole lot. After grinding the full bulk sample, a subsample was taken for the actual analytical process. The feed samples were analyzed for Aflatoxin B_1 (AFB_1), Aflatoxin B_2 (AFB_2), Aflatoxin G_1 (AFG_1), Aflatoxin G_2 (AFG_2), Diacetoxyscirpenol (DAS), OTA, Toxin HT-2, Toxin T-2 and zearalenone (ZON). The feeding of the animals was conducted under practical farming conditions by the dairy farms chosen. The contamination of the feedstuffs was not intentional or controlled, therefore the initial concentration or even the presence of mycotoxins in the samples was not known.

A total of 85 milk samples from cows ($n=21$), sheep ($n=44$) and goats ($n=20$) were collected from the same dairy farms. During this period the nutrition of sheep and goats was based mainly on supplementary feeding apart from the limiting grazing. The sheep grazed on pasture while the goats on rangelands with shrubs and trees. On the other hand the nutrition of cows was based on supplementary feeding with no grazing at all throughout the year. The supplementary feeding of sheep and goats, consisted of alfalfa hay, cottonseed cake, concentrates and corn grain, while that of cows of maize silage, alfalfa hay and concentrates.

One milk sample was taken from the pooled milk of each farm from the milk cooling tank. Additional feed samples were also collected from those dairy farms at the same day with the milk samples. The milk samples were analyzed, apart from the above mycotoxins, also for Aflatoxin M_1 (AFM_1) and Ochratoxin α (OTα).

2.2. Reagents and solutions

The analytical standards AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, DAS, OTA, OTα, Toxin HT-2, Toxin T-2 and ZON were obtained by Sigma-Aldrich (USA). LC–MS grade methanol and water were used. All solvents were obtained from Lab Scan (Ireland) and were HPLC grade. Ammonium formate and formic acid were obtained from Fluka (Buchs, Switzerland).

2.2.1. Preparation of stock standard solutions

Stock standard solutions with concentration ranging from 100 to 500 mg/L were prepared in methanol. The stock standard solutions were stored at −20 °C. A single composite working standard solution was prepared by combining aliquots of each stock solution and diluting in methanol to obtain a final concentration of 1000 μg/L.

2.2.2. Preparation of matrix matched calibration standards (MMCS)

Matrix effects are generally the combined effect of all components of the sample, other than the analyte, on the measurement of the quantity [14]. As to take into account the matrix effect in the measurements, Matrix Matched Calibration Standards (MMCS) were used for quantitation and confirmation. Sample extracts from all matrices were obtained by following the sample extraction procedure as described previously. These extracts were used for the preparation of MMCS. For the preparation of the MMCS, an aliquot of 1 mL of the blank extract of the methanol phase was evaporated to dryness by a stream of N_2 and 1 mL of a standard solution, of the desired concentration, prepared in methanol was added. Before the injection in the chromatographic system the final solution was filtered through a disposable PTFE syringe filters, 0.45 μm.

2.3. Extraction procedure and analysis

The sample preparation procedure is based on an analytical method used for the determination of polar pesticide residues (e.g. glyphosate, ethephon, chlormequat, mepiquat) in products of animal origin [15] minimizing the workload if conducting multi-residue/contaminant controls. Due to the lack of the clean-up step the presence of co-extractives is anticipated, therefore the behavior of the analytes in several different animal feed commodities, such as corn, cottonseed cake, concentrates and alfalfa hay, was investigated separately.

2.3.1. Extraction procedure for animal feed

An aliquot of 2 ± 0.02 g of previously homogenized sample was weighted into a 50 mL PTFE centrifuge tube (Nalgene, Rochester, NY), 4 mL of water HPLC was added and shaken vigorously for 1 min using a vortex mixer. Four milliliter of acidified methanol (1% HCOOH) was added and the tube was shaken vigorously for 1 min using a vortex mixer. The sample was then centrifuged (4000 rpm) for 5 min. An aliquot of the final extract was transferred into an 8 mL glass vial with a Teflon stopper and stored at −20 °C until analysis. Before injection in the chromatographic system the final extract was filtered through a 0.45 μm Acrodisc PTFE disposable syringe filter (Link Lab Ltd.). Following this extraction procedure the concentration C mg/kg of the analytes in the sample corresponds to $4C$ μg/mL of the analytes in the extract.

2.3.2. Extraction procedure for milk

An aliquot of 2 ± 0.02 g of previously homogenized sample was weighted into a 50 mL PTFE centrifuge tube (Nalgene, Rochester, NY), and 4 mL of acidified methanol (1% HCOOH) was added and shaken vigorously for 1 min using a vortex mixer. The sample was then centrifuged (4000 rpm) for 5 min. The supernatant methanol phase was then taken and transferred to a 15 mL centrifuge tube and stored for at least 12 h in the freezer. Freezing out helps to partly remove some additional co-extractives with limited

solubility in methanol while the major part of fat solidify and precipitate. An aliquot of the supernatant was transferred into an 8 mL glass vial with a Teflon stopper and stored at −20 °C until the analysis. Before injection in the chromatographic system the final extract was filtered through a disposable syringe filters, 0.45 μm Acrodisc PTFE (Link Lab Ltd.). Following this extraction procedure the concentration *C* mg/kg of the analytes in the sample corresponds to 2*C* μg/mL of the analytes in the extract.

2.3.3. Determination with LC–MS/MS

Chromatographic separation was achieved using an LC system consisting of an Agilent Series 1200 (Agilent Technologies, Santa Clara, CA, USA) Degaser (G1379B), an autosampler (Hip/ALS G1367A) with a thermostat (FC/ALS Therm G1330B), a binary pump (G1312A) and a thermostated column department (TCC G1316A) equipped with a reverse phase Polaris C_{18} 5 μm particle size, 50 mm × 2 mm analytical column (Varian, Palo Alto, CA, USA), at a flow rate of 250 μL/min with a mobile phase consisting of water with 5 mM ammonium formate, 0.1% formic acid and 0.02% acetonitrile (solvent A) and methanol with 5 mM ammonium formate and 0.1% formic acid (solvent B). A gradient program was used consisting of 70% of solvent A and 30% of solvent B, ramped linearly over the course of 5 min to 100% of solvent B. This composition was held for a further 5 min before returning to the initial condition. The column was re-equilibrated for 5 min at the initial mobile phase composition. The total run-time was 15 min. The injection volume was 50 μL. In order to avoid carry-over the needle was washed in flush port for 10 s with solvent B after each injection.

Detection was achieved using a triple quadrupole mass spectrometer (Agilent Triple Quad 6410) equipped with an electrospray ionization interface operating in positive or negative mode depending on the analyte. Typical source parameters were as follows: fragmentor voltage and collision energy varied depending on the precursor or product ion (Table 1), drying gas temperature was set at 300 °C, drying and nebulizing gas was nitrogen generated from a high purity nitrogen generator (Nitroflow Basic Mobile, Parker Filtration & Separation B.V.) and their values were set at 7 L/min and 30 psi respectively. For the MS/MS mode, nitrogen was used as collision gas with at 1.5 mTorr. The multiple reaction monitoring experiments were conducted with a dwell time of 50–100 ms. For instrument control, Agilent Mass Hunter data acquisition Triple Quad B.01.04 and for data processing Agilent Mass Hunter Workstation Qualitative Analysis B.01.04 were used.

2.4. Method validation

The validation of the method was performed on the basis of the Regulation no. 401/2006 [16] and Commission decision 2002/657/EC [17] and in addition the analytical SANCO guidelines regarding the validation procedures of analytical methods for pesticide residue analysis were also taken into consideration [18,19]. The analytical characteristics evaluated were sensitivity (expressed as limit of quantification and limit of detection), mean recovery (as a measure of accuracy), precision (expressed as repeatability), and specificity.

2.4.1. Selection of representative analytes

The use of representative analytes in the method validation is very common and in other categories such as pesticides in which the physicochemical properties vary and the selection of the specific analytes can be extrapolated to a whole group [18,19].

A full validation was performed in 9 of the 11 analytes studied, due to low availability of sufficient standard quantity of the analytes OTα and AFM_1. The physicochemical properties of these analytes are similar to the OTA, and AFB_1, AFB_2, AFG_1, and AFG_2 therefore are considered to have a similar behavior during the extraction and determination procedure.

In this case for AFM_1, the use of 4 analytes of the same group and for OTα the use of OTA in the validation procedure is considered to be sufficient. The LC–MS/MS system was calibrated as to ensure that the analytes AFM_1 and OTα can be detected at the lowest calibration level and although a full validation was not preformed, additional procedural recoveries at 0.05 μg/kg were conducted. The lower level of 0.05 μg/kg was necessary as to ensure that the method covers the maximum levels, as set by Regulation no. 1881/2006 [20], for AFM_1 in raw milk, heat-treated milk and milk for the manufacture of milk-based products.

Due to the high variability of the samples, the validation procedure was performed in all matrices categories of the samples. Therefore regarding the animal feed matrices, corn, hay, cotton-cake, and concentrated cereal mix were used and regarding milk samples, whole milk was used.

2.4.2. Linearity

Calibration curves were constructed from injections of MMCS in all 4 animal feed matrices, milk and of standards in solvent (methanol) at seven concentrations (1.75–2.5–5–10–20–40–80 μg/mL) for all analytes. Based on the extraction procedure the lowest fortification level (10 μg/kg) will correspond to a minimum concentration of 2.5 μg/L for animal feed and 5 μg/L for milk. Therefore the level of 1.75 μg/L was chosen as the lowest concentration level of the calibration curve as to cover the lower end of the calibration range. For samples that were positive for an analyte but the peak area was outside the highest calibration level, a dilution of the sample was performed as for the peak area to be within the calibration range and a dilution factor was used as to estimate the final concentration (μg/kg) in the sample.

These calibration curves are used to obtain the predicted concentration *C* (mg/kg) of the analyte from a sample which

Table 1
Chromatographic and MS/MS parameters for the analytes studied. The ionization mode was ESI^+ for all analytes and ESI^- for Aflatoxin M1 only.

Analyte	Group	Pseudo-molecular ion	Precursor Ion	Product ion (quantification)	Product ion (qualifier)	Dwell time	Fragmentor (V)	Collision energy (V)
Aflatoxin B1	Aflatoxins	$[M+H]^+$	313	285	241	50	135	45/25
Aflatoxin B2	Aflatoxins	$[M+H]^+$	315	287	259	50	140	20/20
Aflatoxin G1	Aflatoxins	$[M+H]^+$	331	275	245	50	135	25/25
Aflatoxin G2	Aflatoxins	$[M+H]^+$	329	283	243	50	135	45/45
Aflatoxin M1	Aflatoxins	$[M+H]^-$	255	167	211	100	35	25/15
Ochratoxin A	Ochratoxins	$[M+H]^+$	404	241	239	50	60	20/19
Ochratoxin α	Ochratoxins	$[M+H]^+$	329	259	273.1	100	25	25/25
Diacetoxyscirpenol	Trichothecenes	$[M+NH_4]^+$	384	307	289	50	180	5/5
Toxin HT-2	Trichothecenes	$[M+H]^+$	425	263	105	50	180	25/25
Toxin T-2	Trichothecenes	$[M+NH_4]^+$	484.4	305	245	50	110	5/5
Zearalenone	Fusarium toxin	$[M+H]^+$	319	187	185	50	100	5/5

produces an observed response y by the following equation:

$$C = (y - a)/b$$

According to EURACHEM [21] there are four sources of uncertainty to consider in arriving at an uncertainty on the estimated concentration C. The most significant of them for normal practice are due to variability in the peak area y. The uncertainty S_u of C due to variability in y, can be estimated from the calibration data, by the following equation:

$$S_u = \frac{S_{y/c}}{b}$$

were

$$S_{y/c} = \sqrt{\Sigma_i \frac{(y_i - \bar{y})^2}{n-2}}$$

is the residual for the ith point and b is the slope of the regression line and n is the number of the data points in the calibration.

2.4.3. Trueness and precision

Recoveries, repeatability and reproducibility of the analytes were established in order to evaluate the methods' trueness and precision respectively. Mean recoveries of 70–110% for concentrations from 1 μg/kg to 10 μg/kg and 80–110% for concentrations $\geq$ 10 μg/kg [17], with relative standard deviations (RSD) $\leq$ 20% are considered acceptable. However the acceptable range according to EU SANCO [18,19] is wider, reaching 70–120% for mean recoveries, while in certain cases, typically with multiresidue methods, recoveries outside this range may also be acceptable. The mean recoveries were calculated for corn, hay, cottoncake, concentrated cereal mix and milk samples, at two concentration levels.

As to estimate the within laboratory reproducibility, the repeatability experiments at the lowest fortification level, which is the most critical, were repeated in 3 different time periods and the RSD_{wR} was calculated.

For the fortification experiments, animal feed and milk previously analyzed for the absence of mycotoxins were used. The fortified samples were prepared in two levels, 10 μg/kg (1st level) and 100 μg/kg (2nd level), with 5 replicates at each level. In addition, for the analytes AFM_1 and OTα due to a lower desired LOQ, fortified samples in milk at 0.05 μg/kg were also prepared. All measurements were performed with MMCS as to incorporate the matrix effect in the final results.

For the fortification of the samples a fortification standard solution (FSS) was prepared in methanol at 1000 μg/L as described above. A portion of 2 g of animal feed and milk blank samples, were spiked with sufficient amount of the FSS (0.02 mL for the 1st and 0.2 mL for the 2nd level). For AFM_1 and OTα, a FSS, prepared the same day of the experiments, at lower concentration (1 μg/L) was used.

2.4.4. Limit of quantification/determination (LOQ)

The limit of quantification (LOQ) was established as the lowest concentration tested for which recovery and SDr values were satisfactory and with Signal to Noise (S/N) ratio higher than 10. Therefore, as LOQ the lowest validated level with acceptable accuracy and precision results was selected.

2.4.5. Matrix effect estimation

In LC–MS/MS systems, the matrix effect can be attributed to many sources, mainly expressed as ion suppression. In order to estimate if the matrix influences in a significant degree the quantitation of the analytes, the slopes of the calibration lines obtained for each matrix were compared in pairs, using the Student t-test. The t_{cal} is defined in the following equation:

$$t_{cal} = \frac{|b_1 - b_2|}{\sqrt{S_{b1}^2 - S_{b2}^2}}$$

with b_1 and b_2 the slopes of the calibration lines and S_{b1}, S_{b2} the standard deviation of the slopes.

If the theoretical value (t_{theo}) of 2.228 ($df = 7+7-4 = 10$, *two-sided critical region, probability* 95%) exceeds the calculated value t_{cal}, the null hypothesis (that there is no significant difference between the two calibration lines) is accepted.

As to estimate the magnitude of the matrix effect due to the presence of the matrix, paired comparisons between the slopes of the calibration curves of the analytes in solvent and in matrix were compared. The magnitude of the difference (*diff*) expressed in % sensitivity enhancement or reduction was estimated as [22]

$$diff(\%) = (PF - 1)\ 100$$

The parameter factor (*PF*) is calculated with the following equation:

$$PF = \frac{b_1}{b_2}$$

were b_1 and b_2 the slopes of the calibration lines of the analytes respectively.

3. Results and discussion

3.1. Optimization MS–MS parameters

The ionization of the analytes in the positive and negative mode of the electrospray ion source was studied. Table 1 shows the transitions used for quantification and confirmation, the fragmentor voltage/collision energy for each transition and the retention times of the analytes. The compounds are ionized in the form of $[M+H]^+$ or $[M+NH_4]^+$ ions. Tandem mass spectrometry provides a powerful confirmatory tool because it discriminates efficiently between the analyte and the matrix signal. For optimization of the system MS parameters, individual standard solutions at 100 μg/L prepared in methanol were injected at different values of fragmentor voltage (10–300 V) and collision energy (5–200 V). In Figs. 1 and 2 the optimization of the fragmentor voltage and collision energy for the transitions $331 > 275$ and $331 > 245$ of AFG_2 is presented. As shown in Table 1, the optimum fragmentor voltage varies between 35 and 180 V depending on the analyte. Variation of the collision energy influences both sensitivity and fragmentation. The collision energy was optimized for two selective product ions of each precursor ion. The optimum collision energy varies between 5 and 45 V depending on the analyte. Typically, the transition with the maximum sensitivity was selected for quantification. Although all the analytes elute between 5 and 8 min, due to the high selectivity of the MRM a sufficient identification, is achieved. In Fig. 3, a typical chromatogram of all analytes at concentration level 2.5 μg/L is presented.

3.2. Validation of the method

3.2.1. Assessment of linearity

Good linearity was achieved in all cases with correlation coefficients (r) better than 0.9. In most cases the r values were above 0.98. In the case of Toxin HT-2 in corn the r value is 0.93 showing poor linearity, while in the cases of AFB_2, Toxin T-2, and OTA in corn and ZON in corn and cereal mix the r values are between 0.95 and 0.98. Therefore single point calibration is suggested in the case of positing findings with the requirement that the detectors' response of the analyte in the sample extract is

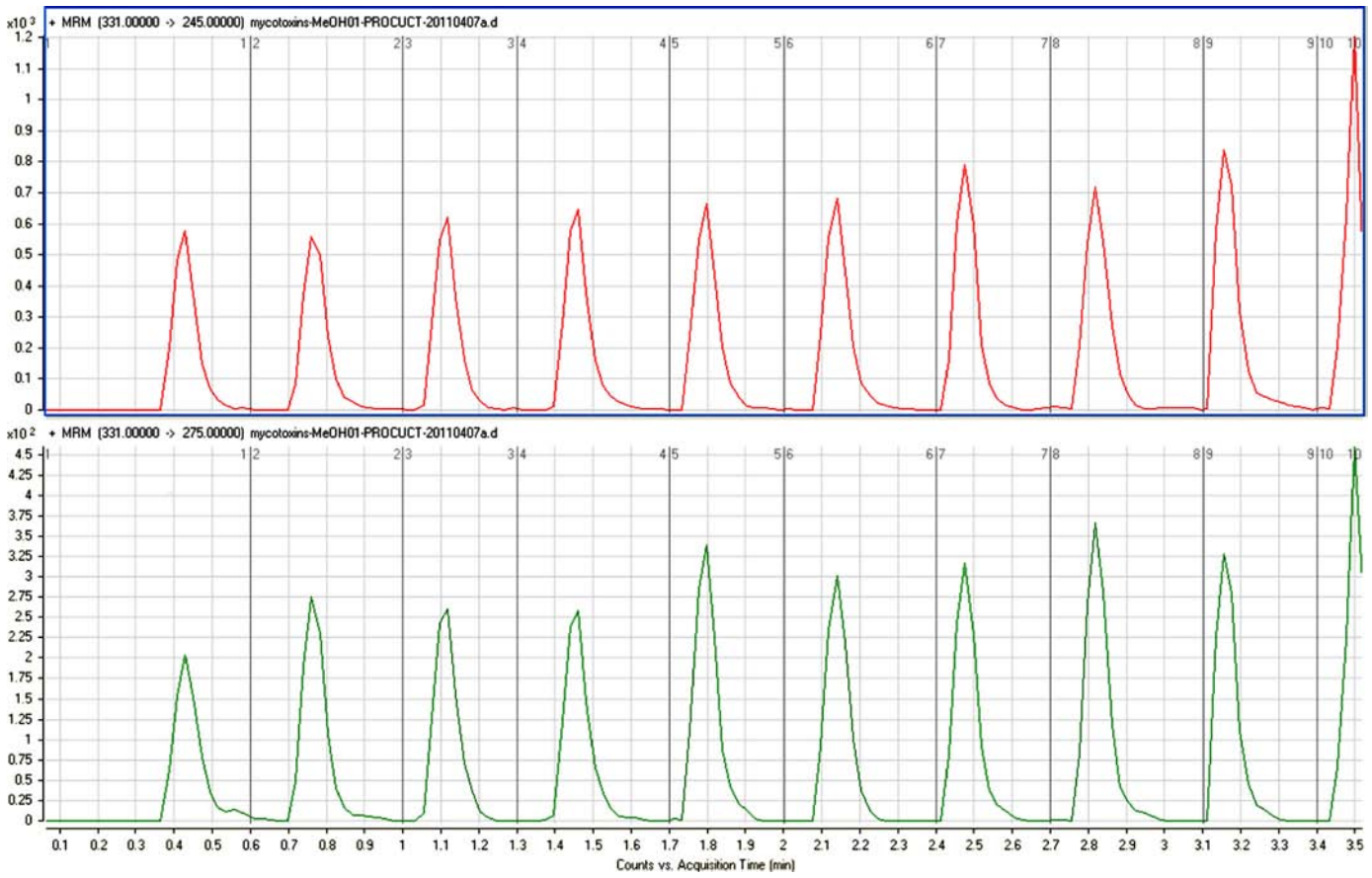

Fig. 1. Repetitive injections for optimization of the fragmentor voltage for the transitions 331 > 275 and 331 > 245 of AFG_2 using different voltages (20–30–40–50–60–70–80–90–100–120–130 V).

within ±30% to the response of the single-calibration matrix matched standard [18,19]. The basic parameters of the regression line are summarized in Table 2.

3.2.2. Trueness and precision

As shown in Table 3, the recoveries of the analytes in animal feed (4 commodities) at the lowest level ranged from 70.6 to 116.2% with RSD values less than 27.2% and at the highest level 70.1–126.9% with RSD values less than 22.7% and RSD_{wR} values less than 27.2%. In milk samples the recoveries ranged from 76.3 to 98.3% with RSD/RSD_{wR} values less than 21.3% and 16.4% respectively at the lowest level and 63.8–115.8% with RSD values less than 18.1% at the highest. In the cases of aflatoxins and DAS in milk the recovery values were below 70%, but consistent (RSD < 11.8%). Therefore, due to the good repeatability/reproducibility, the method is still capable to serve as a quantitative method but when recovery is lower than 70% the final concentration of the analyte in the sample has to be corrected for the recovery.

3.2.3. Limit of quantification/detection (LOQ/LOQ)

The limit of quantification (LOQ) was established as the lowest concentration tested for which recovery and RSD values are satisfactory in accordance with the criteria established for analysis of pesticide residues in foods and with Signal to Noise (*S*/*N*) ratio higher than 10. Therefore, as LOQ the level of 10 μg/kg was set, as it was the lowest validated level with acceptable trueness and precision results. The LOD was set at 1/3 of the LOQ with Signal to Noise (*S*/*N*) ratio higher than 3. In all cases the LOD was set at 3 μg/kg, that covers the EU Maximum Levels for products intended to be used as animal feed [23,24].

At a later stage an expansion of the scope of the method was performed with the addition of AFM_1 and OTα in milk. Therefore, due to sufficient validation data for the group of aflatoxins and Ochratoxin A, a reduced data set of fortification samples (3 replicates) was conducted as to lower the LOQ of the method to 0.05 μg/kg to meet the maximum levels of according to Commission Regulation (EC) no. 1881/2006 [20]. The recoveries values for Aflatoxin M1 and Ochratoxin alpha were 83.3% with RSD 3.5% and 83.7% with RSDr 12.3% respectively.

3.2.4. Assessment of matrix effect

The matrix effect can be attributed to many sources, either in the separation process or the ionization mode of the analyte. As to estimate the effect of the presence of matrix, the calibration curves of the analytes in solvent and in each matrix separately were compared. Significant differences were observed in most cases, between the slopes of the calibration lines meaning that the matrix effect is observed and that quantitation should be conducted with MMCS in order to have reliable and accurate quantitation.

In cereals grain the matrix effect was significant. In all cases except Toxin T-2 and Toxin HT-2 a signal suppression was observed due to the presence of the matrix. The difference between the detectors' response of MMCS between corn and cereal mix showed to be not significant in the cases of AFB2, DAS and Toxin HT-2 indicating that the matrix effect in these anaytes is similar. In all other cases the differences are significant indicating that the nature of the matrix effects differently the detectors response of each analyte. Between cottoncake and cereal

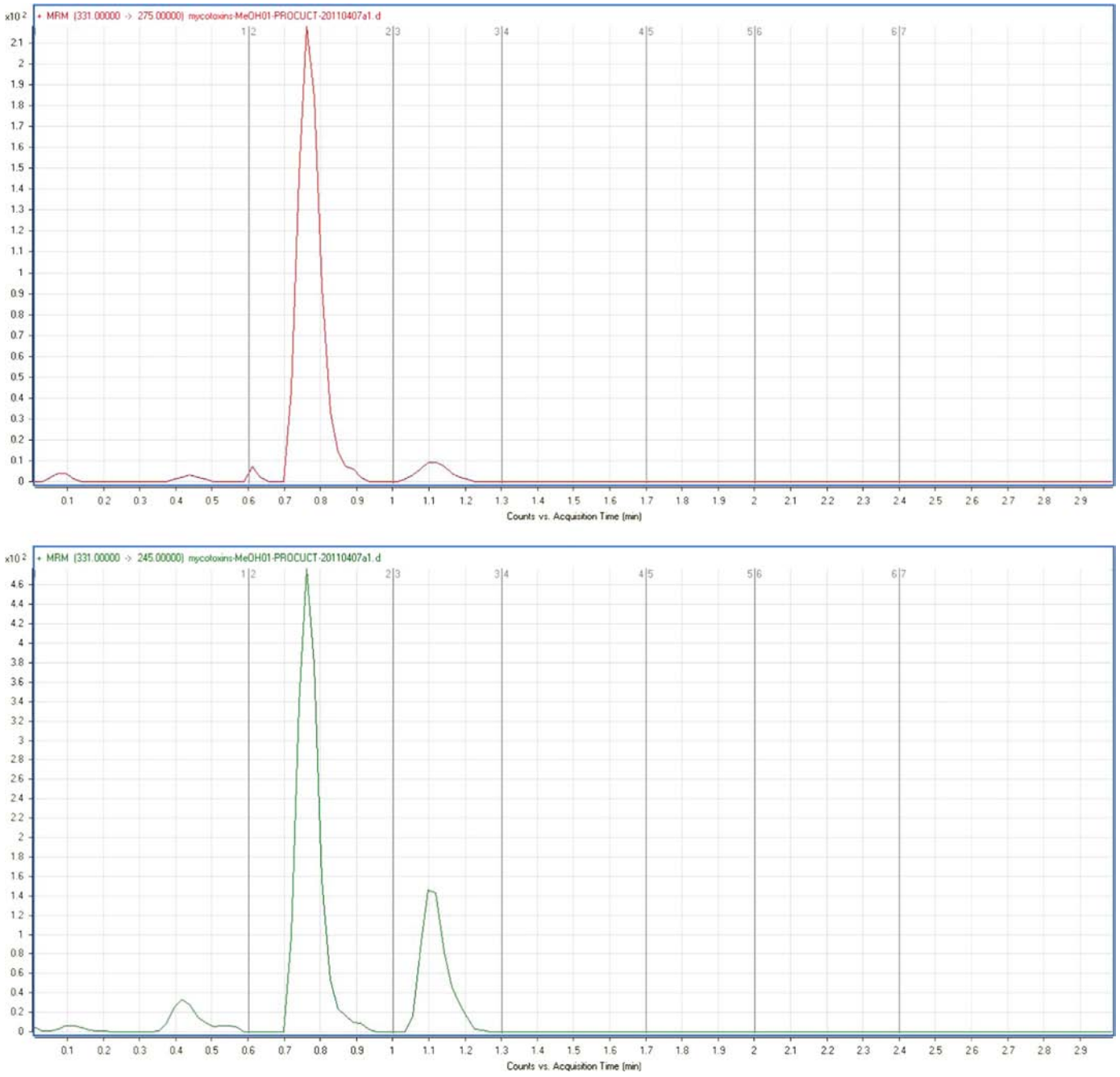

Fig. 2. Repetitive injections for optimizing the collision energy for the transitions 331 > 275 and 331 > 245 of AFG_2 using different voltages (20–40–60–90–120–150 V).

mix, no differences were observed for the analytes Toxin HT-2, AFG2 and Toxin T-2.

In corn, which is also a high oil-dry matrix like cereal grain, the matrix effect was significant in most cases. For the analytes AFB1, Toxin T-2 and ZON no significant effect was observed. The difference between the detectors' response of MMCS between corn and cottoncake showed to be not significant in the cases of Toxin HT-2, OTA and Toxin T-2.

In cottoncake, a high oil commodity and in grass, a high water commodity with low or not relevant concentration of oil, the matrix effect was significant in all cases except Toxin HT-2 and OTA respectively.

Only in the cases of DAS in milk, AFB_1, Toxin T-2, and ZON in corn, and Toxin HT-2 in cottoncake no significant matrix effects were observed. Therefore in the case the method is to be used for targeted analysis on the specific combination of matrix/analyte the quantification can be conducted with calibration standard in solvent as well, without significant error in quantification. If the method is used as a multiclass method for screening or monitoring purposes, MMCS is mandatory. In 72% of the cases the matrix effect was expressed as signal reduction resulting in reduced sensitivity and only in 28% as a signal enhancement, of which only in 19% (corresponding to Toxin HT-2 in corn, milk and cereal mix and Toxin T-2 in milk and cereal mix) this enhancement was significant.

In addition to the estimation of the significance of the presence of any matrix, paired comparisons were conducted between the 4 different matrices of the group of animal feed as to estimate if the nature (in the terms of co-extractives) of the matrix differentiates regarding its effect in the sensitivity of the method. In

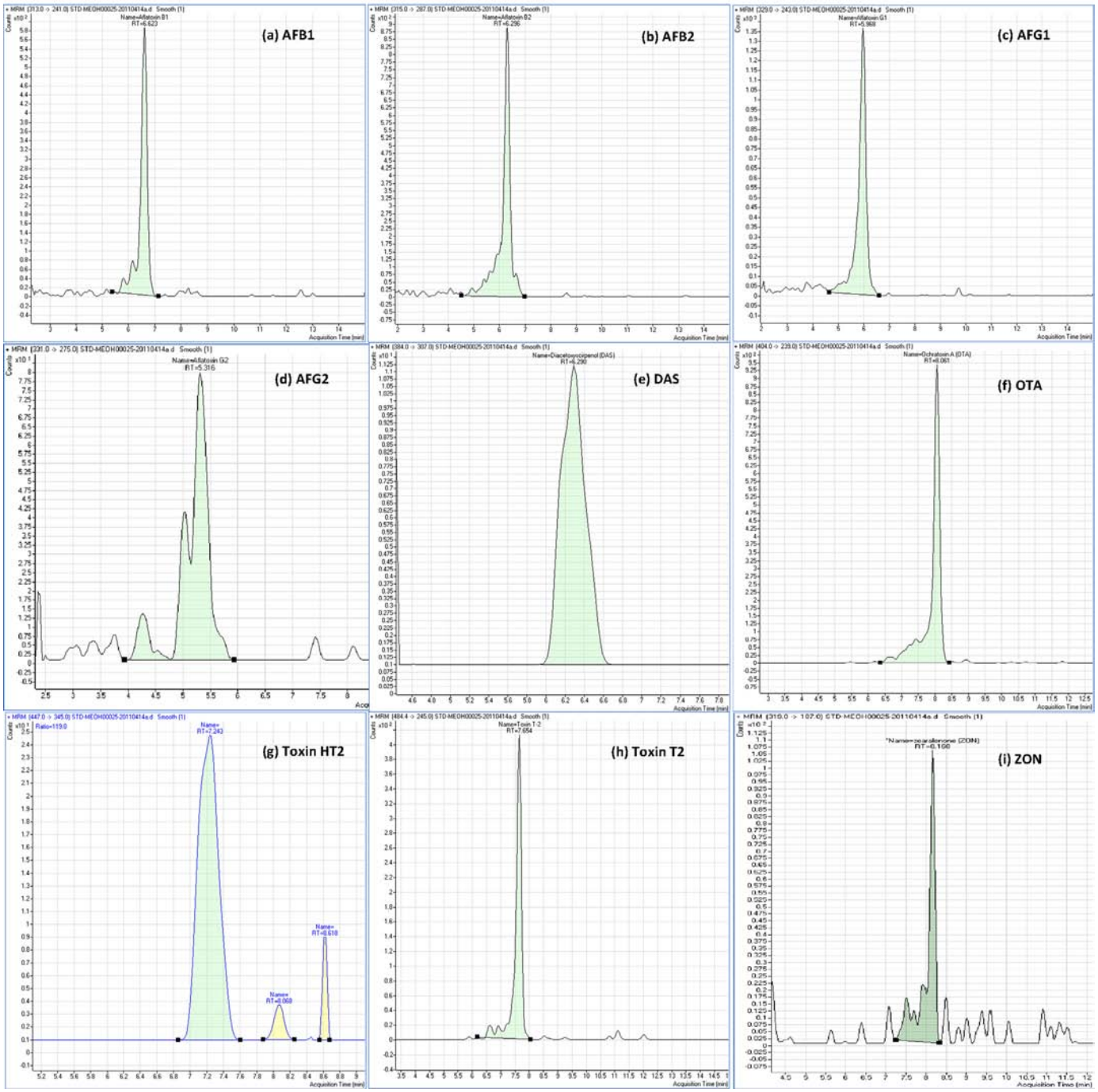

Fig. 3. LC–ESI-MS–MS chromatograms (quantitation transitions) at 2.5 μg/L of all analytes, in methanol: (a) AFB_1, (b) AFB_2, (c) AFG_1, (d) AFG_2, (e) DAS, (f) OTA, (g) Toxin HT-2, (h) Toxin T-2, and (i) ZON.

comparison other matrices to corn, cereal mix and cottoncake presented significant difference in 66.7% of the cases. Only Toxin HT-2 showed to have a similar matrix effect in all matrices. The same pattern can be observed when comparing other matrices to cottoncake. A robustness of Toxin HT-2 in the presence of any matrix can be observed. The individual results for the paired comparison are presented in Table 2.

3.3. *Analysis of feedstuffs and milk samples*

Ruminant diets generally include both forages and concentrates which can increase the probability of contamination with multiple mycotoxins. The first indentified source of mycotoxins in ruminant diets was the contamination of concentrates with aflatoxins. Aflatoxins occur in many typical energy-rich concentrates such as cereal grains, corn gluten, soybean products, as well as in pressed cakes from oil seeds such as sunflower and cotton [11]. Indeed, in this study seven out of 13 cottonseed cake samples had Aflatoxin B_1 (AFB_1) at a level higher than the maximum (5 μg/kg) allowance by EC [25]. The mean AFB_1 concentration (70.71 μg/kg) of the cottonseed cake samples was found to be higher than those have been reported for other feedstuffs by Binder et al. [6] in North Asia (35 μg/kg), South-East Asia (38 μg/kg), South Asia (52 μg/kg) and Oceania (52 μg/kg).

Another, seven out of the 13 cottonseed cake samples were found contaminated with T-2 toxin with values ranged from 8 to 562 μg/kg (Table 4), but no recommendations have been proposed for this

Table 2
Summary of calibration line parameters in milk and animal feed (correlation coefficient r^2, slope of the regression line b, mean standard deviation of the slope of the regression line S_b, mean of the population that corresponds to $x=0$, a mean standard deviation of the mean of the population that corresponds to $x=0$, S_a, standard uncertainty of the concentration S_u), significance of the matrix effect, estimation of the matrix effect and pair comparisons regarding between the matrices compared with corn and cotton cake.

Compound	Matrix	Levels	r	r^2	b	S_b	a	S_a	S_u	Compare with *solvent*		Compare with *corn*	Compare with *cottoncake*
										Sig.	Matrix effect*		
Aflatoxin G_2	Solvent	7	1.000	0.999	8.6E+03	5.2E+01	1.2E+03	8.1E+02	0.42				
	Corn	7	0.977	0.955	1.3E+04	9.4E+02	1.1E+04	7.3E+03	2.3	Sig.	−56		Significant
	Cottoncake	7	0.998	0.995	2.3E+04	4.4E+02	−1.6E+04	6.9E+03	1.4	Sig.	−164	Significant	
	Cereal mix	7	0.996	0.993	2.2E+04	4.0E+02	8.2E+03	6.2E+03	1.2	Sig.	−161	Significant	Not significant
	Milk	7	0.986	0.971	1.6E+04	1.2E+03	4.3E+04	1.8E+04	5.0	Sig.	−90		
	Grass	7	0.999	0.997	6.1E+02	3.3E+01	−1.5E+03	5.2E+02	3.9	Sig.	93		
Aflatoxin G_1	Solvent	7	0.998	0.996	4.1E+04	1.5E+03	6.1E+04	2.3E+04	2.5				
	Corn	7	0.979	0.959	7.0E+04	5.4E+03	6.9E+04	4.2E+04	2.6	Sig.	−70		Significant
	Cottoncake	7	0.997	0.994	9.4E+04	2.3E+03	7.6E+04	3.5E+04	1.7	Sig.	−128	Significant	
	Cereal mix	7	0.999	0.997	8.3E+04	8.9E+02	1.8E+04	1.4E+04	0.7	Sig.	−101	Significant	Significant
	Milk	7	0.987	0.975	7.1E+04	5.3E+03	2.0E+05	8.3E+04	5.3	Sig.	−72		
	Grass	7	0.994	0.989	2.9E+03	5.4E+01	−9.2E+02	8.5E+02	1.3	Sig.	93		
Aflatoxin B_2	Solvent	7	0.999	0.998	3.2E+04	7.6E+02	3.2E+04	1.2E+04	1.7				
	Corn	7	0.974	0.949	4.6E+04	3.9E+03	4.8E+04	3.0E+04	2.8	Sig.	−43		Significant
	Cottoncake	7	0.995	0.990	6.5E+04	1.4E+03	3.3E+04	2.2E+04	1.5	Sig.	−104	Significant	
	Cereal mix	7	0.999	0.998	3.8E+04	3.2E+02	7.8E+03	5.0E+03	0.6	Sig.	−18	Not significant	Significant
	Milk	7	0.988	0.975	4.9E+04	3.5E+03	1.3E+05	5.5E+04	5.0	Sig.	−53		
	Grass	7	0.994	0.988	1.9E+03	1.0E+02	−4.4E+03	1.6E+03	3.8	Sig.	94		
Diacetoxyscirpenol	Solvent	7	0.996	0.991	3.9E+02	8.1E+00	−2.5E+02	1.3E+02	1.5				
	Corn	7	0.986	0.972	5.5E+02	1.9E+01	1.2E+02	1.5E+02	1.1	Sig.	−41		Significant
	Cottoncake	7	0.992	0.984	1.3E+03	3.2E+01	−8.3E+02	5.0E+02	1.8	Sig.	−224	Significant	
	Cereal mix	7	0.996	0.991	5.7E+02	1.8E+01	−7.4E+02	2.9E+02	2.3	Sig.	−48	Not significant	Significant
	Milk	7	0.980	0.960	4.3E+02	2.0E+01	4.1E+02	3.0E+02	3.2	Not sig.	−11		
	Grass	7	0.996	0.992	2.8E+02	1.7E+01	−7.2E+02	2.6E+02	4.1	Sig.	27		
Aflatoxin B_1	Solvent	7	1.000	1.000	2.0E+04	1.8E+02	7.4E+03	2.8E+03	0.7				
	Corn	7	0.999	0.997	1.9E+04	1.8E+02	4.9E+02	1.4E+03	0.3	Not sig.	2		Significant
	Cottoncake	7	1.000	1.000	4.2E+04	6.5E+02	−2.8E+04	1.0E+04	1.1	Sig.	−112	Significant	
	Cereal mix	7	0.998	0.996	2.4E+04	3.0E+02	−8.2E+03	4.7E+03	0.9	Sig.	−20	Significant	Significant
	Milk	7	0.987	0.974	2.9E+04	2.1E+03	8.1E+04	3.3E+04	5.1	Sig.	−49		
	Grass	7	0.966	0.934	1.9E+03	2.7E+02	−1.2E+04	4.2E+03	9.6	Sig.	90		
Toxin HT-2	Solvent	7	0.993	0.985	8.0E+02	5.6E+01	−2.4E+03	8.8E+02	4.9				
	Corn	7	0.930	0.865	6.4E+02	4.2E+01	−1.2E+02	3.3E+02	2.1	Sig.	20		Not significant
	Cottoncake	7	0.978	0.957	7.9E+02	6.0E+01	−2.5E+03	9.3E+02	5.3	Not sig.	1	Not significant	
	Cereal mix	7	0.993	0.985	6.4E+02	4.5E+01	−1.9E+03	7.0E+02	4.9	Sig.	21	Not significant	Not significant
	Milk	7	0.990	0.980	6.4E+02	3.1E+01	1.0E+03	4.8E+02	3.4	Sig.	20		
	Grass**	7	–	–	–	–	–	–	–	–	–		
Toxin T-2	Solvent	7	0.999	0.998	2.5E+04	6.6E+02	2.8E+04	1.0E+04	1.8				
	Corn	7	0.969	0.939	2.9E+04	3.1E+03	4.0E+04	2.4E+04	3.5	Not sig.	−14		Not significant
	Cottoncake	7	1.000	0.999	2.9E+04	1.9E+02	4.3E+03	2.9E+03	0.4	Sig.	−16	Not significant	
	Cereal mix	7	0.999	0.998	1.3E+04	1.1E+02	8.9E+02	1.8E+03	0.6	Sig.	47	Significant	Not significant
	Milk	7	0.985	0.970	1.9E+04	1.5E+03	5.7E+04	2.4E+04	5.6	Sig.	25		
	Grass**	7	–	–	–	–	–	–	–	–	–		
Ochratoxin A	Solvent	7	0.993	0.986	1.2E+03	4.7E+01	1.6E+03	7.3E+02	2.7				
	Corn	7	0.972	0.944	5.7E+04	7.5E+03	1.0E+05	5.8E+04	4.3	Sig.	−4587		Not significant
	Cottoncake	7	0.998	0.995	6.1E+04	1.7E+03	6.4E+04	2.6E+04	1.9	Sig.	−4955	Not significant	
	Cereal mix	7	0.998	0.997	2.1E+04	3.9E+02	1.4E+04	6.1E+03	1.3	Sig.	−1605	Significant	Significant
	Milk	7	0.990	0.980	1.9E+04	1.5E+03	6.0E+04	2.4E+04	5.6	Sig.	−1449		
	Grass	7	0.993	0.987	1.4E+03	3.4E+01	−8,9E+02	5.9E+02	1.7	Not sig.	−5	–	–
Zearalenone	Solvent	7	0.998	0.996	3.7E+03	5.5E+01	1.4E+03	8.7E+02	1.0				
	Corn	7	0.976	0.953	3.7E+03	3,2E+02	4.7E+03	2.9E+03	2.9	Not sig.	1		Significant
	Cottoncake	7	0.999	0.998	5.8E+03	3.2E+02	−2.1E+04	6,4E+03	3.8	Sig.	−58	Significant	
	Cereal mix	7	0.969	0.938	−2.8E+03	1.0E+03	1.1E+05	2.5E+04	−25.7	Sig.	175	Significant	Significant
	Milk	7	0.983	0.966	1.1E+03	9.2E+01	−4.9E+03	3.2E+03	5.8	Sig.	70		
	Grass**	7	–	–	–	–	–	–	–	–	–	–	–

* Negative values is an indication that the analytes present lower sensitivity in the specific matrix than in the matrix (or absence of matrix if diluted in solvent) compared with. The opposite assumption applies for positive values.

** In the case of Toxin HT-2, Toxin T-2, and ZEA due to matrix interferences the linearity could not be acessed.

mycotoxin by the EC. Further to that, in feed samples from Northern, Central and Southern Europe the mean concentrations of T-2 toxin were found to be 137, 190 and 30 μg/kg, respectively [6].

Further to AFB_1 and T-2 toxin, 5 out of 13 cottonseed cake samples, were also contaminated with OTA in concentration of 3–23 μg/kg which is lower than the maximum tolerance limit

Table 3
Average recovery values (recovery), RSD (repeatability) and RSD_{wR} (reproducibility) and of the analytes in animal feeds and milk matrices.

Analyte	Commodity	Fortification level (μg/kg)	Day 1[a] (intra-day)		Day 2[a] (intra-day)		Day 3[a] (intra-day)		All days (inter-day)	
			m, Rec (%)	RSD (%)	*m*, Rec (%)	RSD (%)	*m*, Rec (%)	RSD (%)	*m*, Rec (%)	RSD_{wR} (%)
Aflatoxin G_2	Corn	10	108.5	11.0	93.7	15.7	115.6	18.8	105.9	15.2
	Corn	100	102.2	6.0						
	Cottoncake	10	101.9	11.1	67.7	10.5	80.7	12.9	83.5	11.5
	Cottoncake	100	103.3	5.4						
	Cereal mix	10	107.1	6.0	105.1	13.0	86.5	14.4	99.5	11.2
	Cereal mix	100	93.1	7.3						
	Grass	10	70.1	9.4	95.2	12.0	102.1	12.3	89.1	11.2
	Grass	100	73.2	10.3						
	Milk	10	57.3	0.8	91.1	21.3	104.5	19.6	84.3	13.9
	Milk	100	71.3	2.8						
Aflatoxin G_1	Corn	10	98.7	1.2	91.9	9.3	116.4	7.1	102.3	5.9
	Corn	100	83.4	3.7						
	Cottoncake	10	85.7	10.9	80.6	9.7	116.8	31.3	94.4	17.3
	Cottoncake	100	74.5	7.4						
	Cereal mix	10	72.3	6.4	70.9	10.0	68.6	13.9	70.6	10.1
	Cereal mix	100	84.7	4.8						
	Grass	10	111.9	16.4	104.8	1.9	98.5	12.8	105.1	10.4
	Grass	100	70.3	13.2						
	Milk	10	56.4	2.5	103.1	13.8	69.3	15.9	76.3	10.7
	Milk	100	68.2	8.9						
Aflatoxin B_2	Corn	10	97.8	1.8	81.5	12.3	79.3	4.2	86.2	6.1
	Corn	100	70.8	6.3						
	Cottoncake	10	107.3	2.2	71.8	2.9	122.2	18.5	100.4	7.9
	Cottoncake	100	75.6	3.2						
	Cereal mix	10	85.2	9.9	74.6	4.3	68.4	9.2	76.1	7.8
	Cereal mix	100	71.0	4.4						
	Grass	10	120.9	14.3	108.9	12.9	118.9	5.9	116.2	11.0
	Grass	100	70.1	4.1						
	Milk	10	62.2	11.9	90.9	7.7	92.3	8.4	81.8	9.4
	Milk	100	72.1	1.6						
Diacetoxyscirpenol (DAS)	Corn	10	104.4	10.7	72.6	46.3	103.6	24.6	93.5	27.2
	Corn	100	98,4	21,6						
	Cottoncake	10	97.2	14.5	86.9	7.2	76.7	20.7	86.9	14.1
	Cottoncake	100	89.5	16.7						
	Cereal mix	10	92.7	21.6	110.5	3.3	126.7	5.5	110.0	10.2
	Cereal mix	100	110.4	22.7						
	Grass	10	119.4	13.9	108.8	14.8	101.1	8.7	109.8	12.5
	Grass	100	107.2	11.5						
	Milk	10	64.4	9.8	94.4	9.7	94.5	4.6	84.4	8.1
	Milk	100	74.3	10.9						
Aflatoxin B_1	Corn	10	90.5	5.5	69.2	14.3	74.3	14.4	78.0	11.4
	Corn	100	89.6	10.6						
	Cottoncake	10	85.6	7.9	72.2	11.9	72.8	12.3	76.9	10.7
	Cottoncake	100	73.2	14.1						
	Cereal mix	10	126.2	13.2	85.8	7.2	64.0	19.4	92.0	13.3
	Cereal mix	100	77.0	5.7						
	Grass	10	93.8	16.2	87.8	12.8	101.4	12.9	94.3	14.0
	Grass	100	72.0	11.3						
	Milk	10	52.9	5.2	100.6	9.1	91.5	5.6	81.7	6.6
	Milk	100	63.8	8.9						
Toxin HT-2	Corn	10	72.1	22.6	118.6	14.1	110.9	15.1	100.5	17.3
	Corn	100	78.4	19.3						
	Cottoncake	10	79.5[b]	15.5	87.8	22.8	82.2	17.8	85.0	20.3

	Cottoncake	100	126.9	9.2						
	Cereal mix	10	93.5	18.1	99.2	9.8	100.6	10.0	97.8	12.6
	Cereal mix	100	91.9	20.9						
	Grass	10	106.8	6.8	88.9	16.8	107.1	17.5	100.9	13.7
	Grass	100	76.2	12.1						
	Milk	10	118.9	14.3	96.3	21.2	67.6	12.3	94.2	15.9
	Milk	100	109,2	18,1						
Toxin T-2	Corn	10	87.5	1.9	124.0	8.9	116.0	9.8	109.2	6.9
	Corn	100	77.9	19.6						
	Cottoncake	10	117.4	4.5	80.7	7.0	99.7	7.3	99.3	6.3
	Cottoncake	100	108.1	2.8						
	Cereal mix	10	98.6	13.1	101.7	7.0	112.3	13.5	104.2	11.2
	Cereal mix	100	103.4	5.2						
	Grass	10	98.1	6.8	115.8	13.8	109.5	15.8	107.8	12.1
	Grass	100	119.1	15.3						
	Milk	10	94.6	12.8	106.0	4.9	93.9	9.1	98.2	8.9
	Milk	100	115.8	13.0						
Ochratoxin A (OTA)	Corn	10	102.2	0.9	114.6	6.7	123.1	11.3	113.3	6.3
	Corn	100	103,7	7,7						
	Cottoncake	10	115.7	5.5	90.3	10.8	106.3	7.2	104.1	7.8
	Cottoncake	100	75.4	2.4						
	Cereal mix	10	103.5	4.4	111.9	12.1	102.5	13.7	106.0	10.1
	Cereal mix	100	70.7	3.9						
	Grass	10	81.2	5.3						
	Grass	100	70.9	3.8						
	Milk	10	91.8	19.8	109.9	9.0	93.1	20.4	98.3	16.4
	Milk	100	98.5	12.8						
Zearalenone (ZEA)	Corn	10	102.8	9.0	85.0	13.0	109.0	15.2	98.9	12.4
	Corn	100	102.5	9.2						
	Cottoncake	10	98.2	17.8	77.2	13.9	128.9	7.8	101.4	13.1
	Cottoncake	100	97,9	6,8						
	Cereal mix	10	116.5	18.7	115.8	6.5	97.2	7.2	109.8	10.8
	Cereal mix	100	84.2	22.6						
	Grass	10	106.6	3.9	87.8	5.2	70.9	8.6	88.4	5.9
	Grass	100	73.3	8.0						
	Milk	10	72.6	6.4	108.7	137	1059	11.6	95.7	10.6
	Milk	100	79.6	5.6						
Aflatoxin M1[c]	Milk	0.05	88.3	3.5						
Ochratoxin α[c]	Milk	0.05	83.7	12.3						

[a] The measurements of each batch of fortified samples was performed with a time interval of 1 week.

[b] In the case of Toxin HT-2 in cottoncake the recoveries in the lower level were very high (666.9%) which this is not the case in the highest level. This is assumed to be due to random error in the experimental stage of interference in the system, therefore the precision of the method was not estimated during the initial validation procedure. The recovery experiment was repeated at a later stage only for this combination of matrix/analyte using the multiresidue analytical acquisition method and gave acceptable recoveries.

[c] 3 Replicates.

Table 4
Concentration of mycotoxins (μg/kg) in individual samples of cottonseed cake, maize silage, alfalfa hay, corn grain and concentrates.

Commodity	AFB_1[a,b]	AFB_2[b]	AFG_2[b]	OTA[c]	T-2[d]
Cottonseed cake	167	49	–	23	76
	52	–	9	11	9
	18	–	4	10	562
	83	–	8	19	546
	34	–	6	–	12
	47	–	10	–	8
	–	–	4	–	13
	94	–	-	3	–
Maize silage	–	450	219	–	–
	–	–	6	17	–
	–	–	12	–	–
	–	–	138	–	–
	–	–	891	–	–
	–	–	5	–	–
	–	–	13	–	–
	–	–	13	–	–
	–	–	17	–	–
Alfalfa hay	–	–	–	23	–
	–	–	–	1697	–
	–	–	4	–	–
	–	–	9	–	–
	–	–	–	22	–
	–	–	28	–	–
	–	–	–	94	–
	–	–	–	12	–
	–	–	87	–	–
	–	–	8	–	–
	–	–	13	–	–
	–	–	39	74	–
	–	–	–	14	–
Corn grain	–	–	15	–	–
Concentrates	–	–	11	–	–

"–" is < LOQ.

[a] The max allowance level of AFB1 in cereals and all products derived from cereals including oilseeds and processed products is 2 μg/kg, in maize to be subjected to sorting or other physical treatment is 5 μg/kg and in complete feedstuffs by EU for dairy ruminants is 5 μg/kg [23,24].

[b] The max sum of $AFB_1+AFB_2+AFG_1+AFG_2$ allowance level by EU=4 μg/kg except for maize where is 10 μg/kg [23].

[c] The max allowance level by EU=250 μg/kg [25].

[d] No recommendation provided by the EU [20].

(250 μg/kg) (Table 4). Similarly, Kokić et al. [26] determined OTA contaminated soybean meal (6 out of 7 samples) and sunflower meal (5 out of 7 samples), with values ranging from 2.61 to 5.12 μg/kg and 2.24 to 3.82 μg/kg, respectively, in the region of Vojvodina.

Information cited from different sources depicted that the incidence of OTA contamination is higher in concentrates. However, it may also occur in forages. Indeed, Skrinjar et al. [27] found OTA contamination in maize silage. On the other hand, most recent studies investigating the occurrence of OTA in silage reported no evidence for a significant occurrence [3,28]. Similarly, in this study only in one maize silage sample was detected OTA with value lower than the official acceptable value (Table 4).

Very few data are available concerning aflatoxins contamination in silages. In this study 9 out of 15 maize silages were contaminated with Aflatoxin G_2 (AFG_2) with values higher than the maximum tolerance limit (Table 4). These results are in agreement with those by Cavallarin et al. [29] who found that in maize silages, ensiling either in laboratory silos or in farm scale silos, AFG_2 and AFB_2 were the more abundant mycotoxins than AFB_1 and AFG_1. AFG_1 also detected in 6 out of 13 alfalfa hay samples at a level higher than the maximum allowance by EC [25].

Further to that, 7 out of 42 alfalfa hay samples were also contaminated with OTA with concentrations ranging from 12 to 1697 μg/kg (Table 4). From these samples only one had OTA concentration higher than the maximum allowance level. Accordingly, Skrinjar et al. [27] found that the concentration of OTA varied from traces to 400 μg/kg in different feed samples including forages like hay and dried lucerne. In Dutch dried forages no evidence for the occurrence of OTA was observed in a study [3], while in others OTA was detected only in one out of 201 straw samples [30].

Ruminants are considered more resistant to adverse effects of mycotoxins [11]. This assumption is based on the findings that rumen microorganisms have biotransformation ability of mycotoxins to less toxic or not toxin metabolites [4]. More specifically, Aflatoxin M_1 (AFM_1) is the main hepatic metabolite of AFB_1, while Ochratoxin a (OTα) is the metabolite of OTA. Due to the fact that AFM_1 and OTα are eliminated in urine, feces and milk, in this study all the milk samples were examined for any contamination of these mycotoxins too. However, in this study AFM_1 was not found in levels above the LOD in any of the analyzed milk samples (cows/sheep/goats). Similar results, as the AFM_1 content concerns, have been reported by Saccà et al. [31] for raw milk samples from 17 dairy goat farms from North-eastern Italy, for sheep by Finoli and Vecchio [32] in Western Sicily, and for cows by Boudra et al. [33] in France. On the other hand, in the region of Serbia from raw milk samples (cows=3, sheep=2, goats=7) only the goats milk samples had AFM_1 concentration [34] higher than 0.05 μg/L which is the maximum level allowance by European Union regulation [35]. In a study from Iran, Rahimi et al. [36] when examined raw milk samples of cows (n=75), sheep (n=51) and goats (n=70) found that the 36%, 3.9% and 5.7% of those samples, respectively, had AFM_1 concentrations higher than the maximum tolerance limit. Pathirana et al. [37] in Sri Lanka, when collected 87 raw milk samples from 29 dairies of cows, found that the percentage of contaminated samples, which exceeded the recommended limit, with AFM_1 was 9.2%. Similarly, Delialioğlu et al. [38] in Turkey, when tested 39 and 53 milk samples from goats and cows respectively, induced that in 10.2% of goat milk and in 3.5% of cow milk samples the AFM_1 level was above the official reasonable limit value.

Even though investigations on milk AFM_1 contamination are regularly conducted in EEC countries, there is limited information on the contamination of raw milk by other mycotoxins. However, there is some information concerning the presence of OTA in cows raw milk, while no data are available for sheep and goats milk. To our knowledge this is the first study which tested possible contamination of raw milk from sheep and goats for OTA and OTα. In a number of studies on bovine milk no OTA and OTα were detected in 121 samples from Northern Germany [39]. Further to that, no OTA was found in 100 bovine milk samples in the UK both produced conventionally and organically [40] and in 48 [41] and 12 [42] samples from Spain. On the other hand, in a survey in the Northwest of France, Brouda et al. [33] determined OTA in three out of 264 bovine milk samples at low level, ranging from 5 to 8 ng/L, while OTα was not detected in any sample. In this study, no OTA and OTα were detected above the LOD in any milk sample, possibly due to a very effective degradation in rumen or due to low contamination of the feeds used.

Due to the complex and variable composition of ruminant's diet or even more and from a single feed the animals could be exposed to more than one mycotoxins or mycotoxin cluster; the term "*cluster*" refers to a set of mycotoxins produced by individual fungal species. This has as a result increased the risk of the toxic effects to the animal and increased the concentration of each toxin to the end products and particularly to milk. Indeed, in this study the feeds were exposed to more than one mycotoxin despite that no contamination was observed in the milk samples for the examined mycotoxins. The synergistic or the additives effects of mycotoxins have been studied briefly in pigs and poultry while the

opposite has been done in ruminants where quite often, problems due to subclinical levels of mycotoxins are expressed as minor increases in "common cow problems".

4. Conclusions

In the field of mycotoxin analysis there is a tendency toward simple, cheap and multiresidue analytical methods. The possibility of multi-contaminant analytical methods with few analytical steps that can be used for routine or research purposes is the next step, although this approach is still scarce. In this study an easy, economic and reliable analytical method for the determination of multiclass mycotoxins was developed and validated.

The advantage of this method is the combination of an easy and cheap extraction procedure based on methanol extraction, without any cleanup step. This procedure was based on an analytical method already validated for polar pesticide residues; therefore a simultaneous extraction of these analytes as well is feasible. However a different analytical column (HILIC or ion exchange) is required for the determination of these analytes. The determination of the analytes in a MS/MS system working in MRM mode and combined with LC gave reliable qualification and quantification of the analytes. The method presented acceptable trueness, precision and linearity with an LOQ set generally at 10 μg/kg but 0.05 μg/kg for AFM_1 and OTα in milk. In most cases the matrix effect was found significant and therefore MMCS should be used, as to have more reliable results.

The method was applied to real samples of concentrates and forages, in which, as shown from the results, mycotoxins occurrence can arise in both matrices. More specifically, AFG_2 was the more abundant aflatoxin in maize silage and alfalfa hay samples. The fact that a number of cotton seeds cake samples, further to AFG_2 and AFB_1 aflatoxins, were also contaminated with T-2 toxin underlines the importance to institute tolerance limits by the EC for this myxotoxin too. The results concerning the multi-mycotoxin exposure in ruminant's feeds clearly shows the significance of using an easy, cheap and sensitive method for LC–MS/MS like this which has been developed in this study to analyze a multiple classes of mycotoxins with minimum sample preparation. Finally, the cow, sheep and goats milk from Greece for the sampling collection period was without any risk for human health because none of the tested mycotoxins were detected.

Conflict of interest

None.

References

[1] K.R.N. Reddy, B. Salleh, B. Saad, H.K. Abbas, C.A. Abel, W.T. Shier, Toxin Rev. 29 (2010) 3–26.
[2] S. Sforza, C. Dall' Asta, R. Marcheli, Mass Spectrom. Rev. 25 (2006) 54–76.
[3] F. Driehuis, M.C. Spanjer, J.M. Scholten, M.C. Te Giffel, Food Addit. Contam. B 1 (2008) 41–50.
[4] S.D. Upadhaya, M.A. Park, J.K. Ha, J. Anim. Sci. 23 (2010) 1250–1260.
[5] M. Mobashar, J. Hummel, R. Blank, K.H. Sudekum, Toxins 2 (2010) 809–839.
[6] E.M. Binder, L.M. Tan, L.J. Chin, J. Handl, J. Richard, Anim. Feed Sci. Technol. 137 (2007) 265–282.
[7] N. Sultana, N.Q. Hanif, Pak. Vet. J. 29 (2009) 211–213.
[8] O.O.M. Iheshiulor, B.O. Esonu, O.K. Chuwuka, A.A. Omede, I.C. Okolo, I.P. Ogbuewu, Asian J. Anim. Sci. 5 (2011) 19–33.
[9] J.P. Jouany, A. Yiannikouris, G. Bertin, Cent. Int. Hautes Etudes Agron. Méditerr. A 85 (2009) 205–224.
[10] Commission Directive 2003/100/EC of 31 October 2003 Amending Annex I to Directive 2002/32/EC of the European Parliament and of the Council on Undesirable Substances in Animal Feed Text with EEA Relevance, Off. J. Eur. Commun. L 285 (2003) 33–37.
[11] J. Fink-Gremmels, Food Addit. Contam. 25 (2008) 172–180.
[12] P. Songsermsakul, E. Razzazi-Fazeli, J. Liq. Chromatogr. Relat. Technol. 31 (2008) 1641–1686.
[13] H.G.J. Mol, P. Plaza-Bolaños, P. Zomer, T.C. de Rijk, A.A.M. Stolker, P.P.J. Mulder, Anal. Chem. 80 (2008) 9450–9459.
[14] A.D. McNaught, A. Wilkinson, IUPAC: Compendium of Chemical Terminology The Gold Book, second ed., Blackwell Science Ltd., Oxford, UK, 1997.
[15] M. Anastassiades, D.I. Kolberg, D. Mack, A. Barth, C. Wildgrube, D. Roux, in: E. Commission (Ed.), Food of Animal Origin, CVUA Stuttgart, Stuttgard, Germany, 2013.
[16] Commission Regulation (EC) No. 401/2006 Laying Down the Methods of Sampling and Analysis for the Official Control of the Levels of Mycotoxins in Foodstuffs, Off. J. Eur. Commun. L 70 (9) (2006) 12–34.
[17] Commission Decision 2002/657/EC of 12 August 2002 Implementing Council Directive 96/23/EC Concerning the Performance of Analytical Methods and the Interpretation of Results, Off. J. Eur. Commun. L 221 (2002) 8–36.
[18] E. Commission, Document No. SANCO/12495/2011, Method Validation And Quality Control Procedures For Pesticide Residues Analysis In Food And Feed, 2011, pp. 40.
[19] E. Commission, Document No. SANCO/12571/2013, Method Validation And Quality Control Procedures For Pesticide Residues Analysis In Food And Feed, 2013, pp. 42.
[20] Commission Regulation (EC) No. 1881/2006 of 19 December 2006, Setting Maximum Levels for Certain Contaminants in Foodstuffs, Off. J. Eur. Commun. L 364 (2006) 5–24.
[21] S.L.R. Ellison, A. Williams, EURACHEM/CITAC Guide: Quantifying Uncertainty in Analytical Measurement, 2012.
[22] H. Trufelli, P. Palma, G. Famiglini, A. Cappiellol, Mass Spectrom. Rev. 30 (3) (2011) 491–509.
[23] Commission Regulation (EU) No. 165/2010 of 26 February 2010 Amending Regulation (EC) No. 1881/2006 Setting Maximum Levels for Certain Contaminants in Foodstuffs as Regards Aflatoxins, Off. J. Eur. Commun. L 50 (2010) 8–12.
[24] Commission Directive 2002/32/ EC of the European Parliament and of the COUNCIL of 7 May 2002 on Undesirable Substances in Animal Feed, Off. J. Eur. Commun. L 140 (2002) 10–20.
[25] Commission Directive 2006/576/EC, Commission Recommendation on the Presence of Deoxynivalenol Zearalenone Ochratoxin A T-2 HT-2 and Fumonisins in Products Intended for Animal Feeding, Off. J. Eur. Commun. L 229 (2006) 7–9.
[26] B.M. Kokić, I.S. Čabarkapa, J.D. Lević, A.I. Mandić, J.J. Matić, D.S. Ivanov, Proc. Nat. Sci. 117 (2009) 87–96.
[27] M. Skrinjar, R.D. Stubblefield, I.F. Vujicic, Acta Vet. Hung. 40 (1992) 185–190.
[28] E. Richard, N. Heutte, V. Bouchart, D. Garon, Anim. Feed Sci. Technol. 148 (2009) 309–320.
[29] L. Cavallarin, E. Tabacco, S. Antoniazzi, G. Borreani, J. Sci. Food Agric. 91 (2011) 2419–2425.
[30] S. Sondermann, M. Schollenberger, W. Drochner, D. Rohweder, H. Valenta, S. Dänicke, K. Hartung, H.P. Piepho, Proc. Soc. Nutr. Physiol. 19 (2010) 147.
[31] E. Saccà, D. Boscolo, A. Vallati, W. Ventura, F. Bigaran, E. Piasentier, J. Sci. Food Agric. 89 (2009) 487–493.
[32] C. Finoli, A. Vecchio, Ital. J. Anim. Sci. 2 (2003) 191–196.
[33] H. Boudra, J. Barnouin, S. Dragacci, D.P. Morgavi, J. Dairy Sci. 90 (2007) 3197–3201.
[34] M. Polovinski-Horvatović, V. Jurić, D. Glamočić, Biotechnol. Anim. Husb. 25 (2009) 713–718.
[35] Commission Directive 2001/466/EC of 8 March 2001 Setting Maximum Levels for Certain Contaminants in Foodstuffs, Text with EEA Relevance, Off. J. Eur. Commun. L 77 (2001) 1–13.
[36] E. Rahimi, M. Boniadian, M. Rafei, H.R. Kazemeini, Food Chem. Toxicol. 48 (2010) 129–131.
[37] U.P.D. Pathirana, K.M.S. Wimalasiri, K.F.S.T. Silva, S.P. Gunarathne, Trop. Agric. Res. 21 (2010) 119–125.
[38] N. Delialioğlu, F. Otağ, N.D. Ocal, G. Aslan, G. Emekda, Bull. Microbiol. 44 (2010) 87–91.
[39] H. Valenta, M. Goll, Food Addit. Contam. 13 (1996) 669–676.
[40] Food Standards Agency, Survey of Milk for Mycotoxins Number 17/01-Food Survey Information Sheet, UK, 2001.
[41] L. González-Osnaya, J.M. Soriano, J.C. Moltó, J. Manes, Food Chem. 108 (2008) 272–276.
[42] A. Bascarán, A. Hernández de Rojas, P. Choucino, T. Delgado, J. Chromatogr. A 1167 (2007) 95–101.