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Occurrence of mycotoxins in total mixed ration of dairy farms in Portugal and carry-over to milk

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ABSTRACT

This study aims to explore the presence of mycotoxins in total mixed rations (TMR) employed for feeding dairy cattle and their potential carry-over to milk. A total of 87 TRM samples were collected in farms from the north of Portugal from 2019 to 2022. A method based on a QuEChERS extraction followed by UHPLC-MS/MS detection was employed for sample analysis. The method was in-house validated in terms of matrix effect, linearity, sensitivity, accuracy and precision. The most frequently detected regulated toxins were fumonisins, with a positivity rate of 74%, while deoxynivalenol and zearalenone were found in approximately 35% of the samples. Among the emerging toxins, beauvericin and enniatins exhibited the highest detection rates. In addition, milk samples were collected from 21 farms, providing insights into the carry-over of these toxins and roquefortine to milk, with an estimated rate ranging between 2% and 10%.

1. Introduction

In the European Union (EU), dairy production is the second-biggest agricultural industry. Over the past 20 years, there has been a progressive change in the dairy production landscape, with a remarkable 50% rise in the milk yield per dairy cow (Karlsson et al., 2023). This achievement was accompanied by a shift towards intensified feeding systems, involving the partial or complete substitution of grazing. This was possible through the implementation of balanced rations, commonly known as Total Mixed Rations (TMR). TMR are produced by blending different proportions of forages, byproducts, concentrates, minerals, vitamins, and additives. This comprehensive mixture is formulated to provide a balanced and nutritionally complete diet for livestock, meeting their specific needs for maintenance and production (Schingoethe, 2017).

One of the main risks associated with TMR is that most of their constituents are susceptible to mycotoxin contamination (Garcia et al., 2023). Mycotoxins are fungal metabolites that evoke a toxic response when introduced in low concentration to higher vertebrates and other animals by a natural route (Bennett, 1987). The most significant groups of mycotoxins, such as aflatoxins, ochratoxin A (OTA), deoxynivalenol (DON), fumonisins, or zearalenone (ZEN), are regulated in many nations throughout the world (Sainz et al., 2018). However, there are many other fungal metabolites, not currently subject to legislative regulations despite their known toxicity, which are known as emerging mycotoxins. According to their frequency of occurrence and concentrations, the most significant compounds in this group include, among others, alternariol (AOH), enniatins, beauvericin (BEA), sterigmatocystin (STG), mycophenolic acid (MPA), and alternariol monomethyl ether (AME) (Gruber-Dorninger et al., 2017; Pérez-Fuentes et al., 2022).

While the composition of TMR may differ significantly among farms, in Portugal, maize silage is usually their main constituent (Garcia et al., 2023). In a recent study, it was discovered that more than 82% of maize silage samples exhibited the simultaneous presence of DON and BEA. Additionally, there were *Penicillium* and *Aspergillus* toxins, such as STG or roquefortine C (RC), which were found in up to 5% and 12% of samples, respectively (González-Jartín et al., 2022). The use of contaminated silage implies the transfer of mycotoxins to TMR. In this

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context, the presence of mycotoxins in the diet of dairy cows is of particular concern. Although ruminants are generally considered to have lower susceptibility to mycotoxins due to the capacity of the rumen microbiota to degrade these substances, the economic implications remain substantial. In this sense, mycotoxins are associated with decreased livestock production and elevated veterinary care costs. Furthermore, there is a potential transfer of mycotoxins to milk, posing a possible risk for human consumption. Although aflatoxin M_1 (AFM_1) is still a major concern in this food, new observations have shown that milk consistently has low levels of enniatins and beauvericin (BEA) (González-Jartín, Rodríguez-Cañás et al., 2021; Zain, 2011).

Currently, there is limited data on the presence of mycotoxins in TMR. Although various analytical methods have been employed in different regions to assess the presence of these contaminants in TMR, most of them have been developed to analyze one or a few toxins. In China, a high-performance liquid chromatography with fluorescence detection (HPLC-FLD) method was utilized for the analysis of aflatoxin B_1 (AFB_1), revealing the presence of this mycotoxin in all samples (Xu et al., 2021). A similar approach was used in Mexico, where aflatoxins were also detected in all samples, with an average concentration of 26.0 g/kg in the forage incorporated into TMR (Álvarez-Días et al., 2022). The same technique was applied in Lithuania to analyze AFB_1, ZEN, and T-2 toxin, being detected in 49%, 52%, and 55% of the 119 analyzed samples, respectively (Vaičiulienė et al., 2021). In this country, another small survey was conducted using lateral flow tests to analyze ZEN and DON in 12 samples, being detected in 11 and 7, respectively (Falkauskas et al., 2019). ELISA kits were employed in Jordan to analyze aflatoxins, T-2 toxin, DON, ZEN, and fumonisins, highlighting a high occurrence of T-2 toxin (Bani et al., 2020). Another study in Iran, using an ELISA method, found AFB_1 in all analyzed samples (Shad et al., 2019). The most comprehensive approach utilized so far has been a liquid chromatography coupled to mass spectrometry (LC-MS) method to detect mycotoxins, phytoestrogens, and other metabolites. In this way, ZEN, fumonisin B_1 (FB_1), and DON were detected in more than 84% of samples, while AFB_1 and OTA, previously reported in Mexico, were not detected (Penagos-Tabares, Sulyok, et al., 2023).

The presence of mycotoxins in animal feed poses a significant public health concern, as these contaminants enter the food chain, inducing toxicity in animals. Additionally, they have the potential to lead to undesirable residues in animal-derived food products, including milk, meat, or eggs (González-Jartín et al., 2023). Nowadays, dairy cows are housed in intensive farming systems, where they are provided with TMR as their primary feed (Schingoethe, 2017). However, not much is known about the presence of mycotoxins in this matrix. In this context, the objective of this study was to validate a method capable of analyzing both regulated and emerging mycotoxins in TMR. The secondary goal was to investigate the prevalence of these mycotoxins in dairy farms located in Portugal from 2019 to 2022. Furthermore, the study aimed to establish any potential transfer of toxins from TMR to milk.

2. Materials and methods

2.1. Chemicals and reagents

Chemical reagents, including methanol, acetonitrile (ACN), glacial acetic acid (100%), anhydrous magnesium sulfate ($MgSO_4$), and sodium chloride (NaCl), were supplied by Panreac Quimica S.A. (Barcelona, Spain). Formic acid and ammonium formate were procured from Merck (Madrid, Spain) and Fluka (Buchs, Switzerland), respectively. Water was purified in a Millipore Milli-Q Plus system (Millipore, Bedford, MA). Millipore also provided the Ultrafree-MC Durapore membrane centrifugal filters with a 0.22 μm pore size.

Sigma (Madrid, Spain) provided the solid standards, including DON, ZEN, FB_1, AFB_1, aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), RC, gliotoxin (GLIO), fusaric acid (FA), enniatin A (ENNA), enniatin A_1 ($ENNA_1$), enniatin B (ENNB), enniatin B_1 ($ENNB_1$) and BEA. OTA standard was from Laboratorios CIFGA S.A. Romer Labs (Tulln, Austria) supplied the analytical standards, including T-2 toxin, HT-2 toxin, neosolaniol (NEO), fumonisin B_2 (FB_2), citrinin (CTN), alternariol (AOH), alternariol methyl ether (AME), deepoxy-deoxynivalenol (DOM-1), diacetoxyscirpenol (DAS), T-2 triol, hydrolyzed fumonisin B_1 (h-FB_1), mycophenolic acid (MPA), and STG.

2.2. Sampling

Between November 2019 and April 2022, 87 TMR samples were taken from intensive dairy farms in Vila do Conde, North Portugal. The sampling distribution was as follows: in 2019, 33 samples were collected during the Autumn season. In 2020, 3 samples were collected in Autumn, and 16 in Winter. In 2021, 9 samples were collected in Winter, and 15 in Spring. In 2022, 2 samples were collected in Autumn, 5 in Winter, and 4 in Spring. Each sample, weighing 2 kg, was manually gathered during discharge from at least 10 points of fence line feeders. After that, the samples were packed in plastic bags and kept at 4 °C until they were delivered to the laboratory. Twenty-four hours after feeding with the studied TMR, milk samples were taken from the milk cooling tank and stored at −20 °C until analysis.

2.3. LC-MS detection

A 1290 infinity ultra high-performance liquid chromatography (UHPLC) system interfaced to a 6460 triple quadrupole mass spectrometer (Agilent Technologies, Waldbronn, Germany) was used to measure the amount of mycotoxins in TMR samples. The detection conditions had been previously optimized for the analysis of feed and milk (González-Jartín, Alfonso et al., 2021; González-Jartín, Rodríguez-Cañás et al., 2021). Briefly, the separation process employed a Waters ACQUITY HSS T3 column with dimensions of 100 mm × 2.1 mm (inside diameter) and a particle size of 1.8 μm (Waters, Milford, MA), maintaining a temperature of 40 °C. The mobile phases consisted of water containing 0.1% formic acid and 5 mM ammonium formate (mobile phase A), and methanol (mobile phase B). The elution gradient was as follows: an initial hold time of 0.5 min at 0% B was followed by an increase to 14 % B within 0.5 min, and maintained for 1.5 min. Subsequently, the eluent B percentage was elevated to 60% within 1 min and held for 0.5 min. The gradient then progressed to 100% B within 4.5 min, and this composition was kept for 2 min. Finally, a shift to 0% B occurred within 0.5 min, maintaining this percentage for 2.5 min. The flow rate was set at 0.3 mL/min, and a 5 μL injection volume was employed.

The Agilent 6460 triple quadrupole mass spectrometer was configured with an electrospray ionization source (ESI) employing Agilent Jet Stream Technology. The ion source parameters were set as follows: capillary voltage at 4000 V, positive nozzle voltage at 1500 V, negative nozzle voltage at 0 V, nebulizer pressure at 45 psi, sheath gas at 12 L/min and 400 °C, and nebulizer gas at 8 L/min and 350 °C. For each mycotoxin, the fragmentor voltage (FV), cell accelerator voltage (CAV), collision energy (CE), and mass transitions were individually optimized using MassHunter Optimizer software (Table S1).

2.4. Mycotoxin extraction in TMR

The samples underwent a comprehensive homogenization process, following which a 2.5 g portion was accurately measured and placed into a 50 mL Falcon tube and submitted to an extraction procedure previously optimized for silage (González-Jartín et al., 2022). In this sense, samples were subjected to extraction with 10 mL of acetic acid (1%) through vigorous shaking for 5 min using a vortex mixer (Nahita Blue, Auxilab, Spain) at 2500 rpm. Next, 20 mL of acetonitrile (ACN) were introduced, and the stirring process was repeated. Following this, 8 g of $MgSO_4$ and 2 g of NaCl were incorporated, and the mixture was stirred for 1 min. Then, samples were centrifuged at 3134×*g* for 10 min,

and the upper portion of the extract was transferred to a new tube. Finally, 100 μL of the extract were evaporated to dryness and subsequently reconstituted with 400 μL of the sample solvent, consisting of ACN/water/acetic acid [49:50:1 (v/v/v)]. Before UHPLC-MS/MS analysis, aliquots were filtered by using 0.22 μm centrifugal filters.

2.5. In-house validation of the analytical procedure

The validation of the method encompassed key parameters such as linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, and precision. To initiate the validation process, calibration curves were constructed at nine different points using both solvent and TMR extracts. The concentration ranges were from 1.5 to 192 μg/kg for aflatoxins and 50–12800 μg/kg for most of other toxins. These curves were used to calculate the matrix effect and linearity, which were measured by the correlation coefficient (R). Signal suppression/enhancement (SSE) was calculated to express the matrix effect in accordance with the SANTE/11312/2021 guidelines (SANTE, 2021). This was done by comparing the slopes of curves created in both solvent and blank extracts of TMR. The SSE was determined using the following formula: $SSE(\%) = 100 \times \frac{\text{slope of spiked extracts curve}}{\text{slope of standards curve in solvent}}$. The coefficient of variance (CV) was calculated by measuring three consecutive batches of calibration curves constructed in TMR extracts.

In compliance with EU-RL guidelines (Wenzl et al., 2016), blank extracts were analyzed and the following formulas were used to determine the LOD and LOQ: LOQ = 3.3 × LOD, and LOD = 3.9 × Sb/m. In this case, m denotes the slope of the calibration curve made in the sample solvent, and Sb stands for the standard deviation of the noises seen in 10 blank samples.

Accuracy and intra-day precision were determined based on the Eurachem and SANTE guidelines and previous validation procedures (Eurachem, 2014; SANTE, 2021; Sulyok et al., 2006). However, the number of required samples by the guidelines was reduced, and the recovery was evaluated from three replicate blank samples (n = 3) spiked at a single contamination level: 4.8 μg/kg for aflatoxins, 640 μg/kg for CTN, and DOM-1, and at 320 μg/kg for other compounds. The calculation of the amount of each analyte in the extracts relied on solvent-based calibration curves. This approach facilitated the assessment of apparent recoveries (R_A) and relative standard deviation (RSD). R_A was determined by comparing the measured concentration in the spiked samples with the expected concentration according to the following equation $R_A(\%) = 100 \times \frac{\text{area spiked sample}}{\text{area standard}}$. The RSD provided insight into the precision of the method by indicating the variability within the replicate samples. Furthermore, the recovery of the extraction (R_E) was assessed by applying the SSE to the R_A as follows: $R_E(\%) = 100 \times \frac{R_A}{SSE}$, offering an overall measure of the efficiency of the extraction process.

3. Results

3.1. Method validation for TMR analysis

The study of linearity involved the creation of calibration curves at nine concentration levels. R values were higher than 0.995 within the investigated concentration range. Subsequently, LODs and LOQs were calculated, as indicated in Table 1. LOQs were found to range from 0.9 μg/kg for AFB_1 to 287.6 μg/kg for DOM-1, and the repeatability within-batch, expressed as the CV%, showed a variation lower than 5% for most toxins.

The matrix effect was evaluated through the SSE (Table 1), which varied from 37% for HT-2 to 147% for NEO. In general, the matrix caused signal variations lower than 25%, with most of the toxins showing values in the range of 78–125%. Type A trichothecenes, namely DON and DOM-1, showed factors of 53.1% and 65.1%, respectively; whereas fumonisins showed no matrix effect. Similarly, variations lower than 13% were observed for ENNs and BEA. Next, the recovery (R_E) was studied, and the obtained data are shown in Table 1. Recoveries from TMR ranged from 61% for RC, STG and NEO to 95% for GLIO; however, the R_E for CTN was 40%. The precision was high for most mycotoxins, with RSD values lower than 5%. Exceptions were AFG_2, FB_1, HT-2 toxin, and h-FB_1, for which RSD values were higher but always below 17%.

Table 1
Performance characteristics of the analysis method.

Toxin	LOD (μg/kg)	LOQ (μg/kg)	Coefficient of variance (CV, %)	Matrix effect (SSE, %)	Apparent recovery (R_A, %)	Precision (RSD, %)	Accuracy (R_E, %)
AFB_1	0.27	0.9	4.7	82.8	62.6	5.9	75.6
AFB_2	0.30	1.0	3.4	79.1	59.2	3.7	74.8
AFG_1	0.36	1.2	3.3	72.8	52.3	6.9	71.8
AFG_2	0.39	1.3	2.3	71.4	52.1	11.9	72.9
DON	21.0	69.9	5.3	53.1	36.7	0.5	69.2
FB_1	20.4	67.9	3.5	101.8	83.8	11.3	82.4
FB_2	25.4	84.6	4.9	103.1	67.3	4.3	65.2
CTN	78.3	260.6	0.5	124.3	49.8	4.7	40.0
OTA	10.3	34.2	4.8	95.6	63.8	3.2	66.7
T-2 toxin	9.6	31.9	4.7	95.2	62.4	5.6	65.5
HT-2 toxin	9.9	33.0	2.2	37.3	29.9	10.0	80.4
ZEN	7.1	23.5	1.6	98.4	63.9	2.8	64.9
AOH	29.2	97.3	2.3	74.9	47.3	5.4	63.1
AME	6.5	21.6	2.1	85.0	55.2	2.9	64.9
BEA	2.2	7.2	4.4	92.6	60.7	2.4	65.4
ENNA	0.5	1.6	5.7	87.3	67.9	4.4	77.9
$ENNA_1$	0.6	2.0	3.9	88.1	55.0	2.0	62.5
ENNB	1.5	5.0	3.5	97.1	59.9	5.2	61.7
$ENNB_1$	1.1	3.7	5.0	91.7	58.6	2.7	63.9
FA	39.2	130.5	10.9	59.4	39.8	2.3	67.0
DOM-1	86.4	287.6	6.4	65.1	51.5	5.5	79.1
DAS	12.6	42.1	2.3	116.7	84.5	8.0	72.4
T2 triol	22.2	73.9	6.4	46.2	33.5	7.2	72.4
NEO	21.1	70.3	4.1	147.5	90.6	6.1	61.5
h-FB_1	24.3	80.8	12.7	117.4	90.2	17.4	76.8
MPA	17.9	59.6	2.2	108.8	82.0	3.2	75.4
RC	0.5	1.6	5.6	40.1	24.6	3.6	61.4
STG	0.6	2.0	3.0	79.2	48.7	1.9	61.4
GLIO	14.8	49.4	2.0	55.6	52.8	5.9	94.9

3.2. TMR analysis

Complete analysis results are shown in Table S2. A total of 16 mycotoxins were detected in the samples collected over the 4 years of the study. As shown in Table 2, the occurrence varied significantly. AFB_1 was identified in 2% of samples, followed by OTA, detected in 9%. DON and ZEN were found in approximately one-third of the samples, while fumonisins were more frequently detected, occurring in 74% of cases. Regarding emerging compounds produced by *Fusarium* species, such as BEA and enniatins, they were present in up to 88% of the samples. Compounds produce by *Aspergillus* or *Penicillium,* such as MPA, STG, and RC, were detected in 9%, 18%, and 31% of samples, respectively. The only *Alternaria* toxin detected was AME, present in 14% of the samples.

As shown in Fig. 1 and Table 3, 2019 exhibited the lowest prevalence for the most common toxins over the years, with exceptions for ZEN observed in 2020 and DON in 2021. Compared with previous years, 2022 showed a high prevalence of DON (81%), RC (81%) and ZEN (100%), with double the average of 2019–2021. However, the most frequently detected mycotoxins were FB_1, enniatins, and BEA, as they were present in more than 80% of the samples since 2020. As shown in Fig. 2 and Table 3, over the four years, the frequency of ZEN in winter (26%) was lower than in autumn (37%) and spring (63%). Fumonisins, enniatins, and BEA exhibited a similar pattern, with low positivity in autumn (below 50%, except for BEA at 72%), increasing to over 90% in winter, and slightly decreasing in spring. On the other hand, DON and RC displayed unique patterns: nearly 50% of autumn samples were positive for DON, decreasing to 40% in winter and 25% in spring, while RC showed the opposite pattern.

Toxin concentrations, expressed as μg/kg, are shown in Tables 2 and 3. AFB_1 was found with a maximum concentration of 2.9 μg/kg. The median concentrations of DON and FB_1 were similar, around 220 μg/kg, though FB_1 showed higher variability with a maximum concentration of 3541 μg/kg. ZEN and OTA were present at lower amounts, with median concentrations of 68 μg/kg and 36 μg/kg, respectively. However, in some cases, the concentration of ZEN was high, reaching up to 817 μg/kg. Concerning the most prevalent emerging toxins, such as BEA and enniatins, their mean concentrations were lower, at 38 μg/kg and 96 μg/kg, respectively. Less frequent toxins such as RC and MPA appeared in some samples in very high amounts, particularly RC, with one sample containing 15,144 μg/kg.

3.3. Carry-over to milk

Regulated mycotoxins, such as DON, ZEN, OTA, and fumonisins were detected in several TMR samples, however, none were identified in the corresponding milk samples (Table S3). Moreover, no transfer was observed for AME, STG, or MPA. On the contrary, a carry-over was established for RC, BEA and enniatins. In the case of RC, it was identified individually in TMR, with an average concentration of 609 μg/kg, and in milk, average 5.83 μg/kg. However, on three farms, this toxin was detected simultaneously in both TMR and milk. The average consumption was 17.38 mg of RC, with an excretion of 0.49 mg, resulting in a transfer rate of 2.8%. Regarding BEA, the transfer rate was 1.06%, while for ENNA, $ENNA_1$, ENNB, and $ENNB_1$ were 4.85%, 6.66%, 10.10%, and 5.54%, respectively.

4. Discussion

4.1. Method validation for TMR analysis

A method of extraction that was originally created for the analysis of mycotoxins in maize and grass silage was now validated for TMR analysis (González-Jartín et al., 2022). The method demonstrated satisfactory linearity, $R > 0.995$, and high repeatability within-batch. The obtained LOQs allow the quantification of regulated mycotoxins below legal limits or recommendations (European Commission, 2006, 2011).

Table 2
Contamination levels of the studied samples.

(μg/kg)	AFB_1	DON	ZEN	FB_1	FB_2	OTA	ENNA	$ENNA_1$	ENNB	$ENNB_1$	BEA	FA	AME	RC	STG	MPA
Median	2.8	220.1	68.2	218.2	129.0	36.0	4.6	5.5	15.7	7.0	51.3	131.1	19.8	107.6	13.3	98.9
Mean	2.8	293.2	108.1	328.9	141.2	49.6	6.1	6.7	37.9	15.9	96.4	140.3	19.2	1752.0	159.5	375.8
25% Percentile	2.8	146.4	58.7	152.2	83.0	20.7	1.8	3.4	8.7	5.6	26.5	91.3	8.8	51.3	2.4	54.1
75% Percentile	2.9	452.8	106.9	307.7	173.2	87.9	9.3	9.7	64.7	9.6	143.6	172.0	26.5	1010.0	23.9	735.5
Maximum	2.9	735.5	817.8	3541.0	416.3	99.3	22.2	32.7	186.2	135.2	1485.0	275.9	42.6	15144.0	1916.0	1579.0
Detection rate (%)	2.3%	42.5%	39.1%	74.7%	59.8%	9.2%	27.6%	40.2%	71.3%	58.6%	88.5%	19.5%	13.8%	31.0%	18.4%	9.2%

Concentration levels are expressed as μg/kg, and the detection rate is the percentage of samples above the LOQ shown in Table 1.

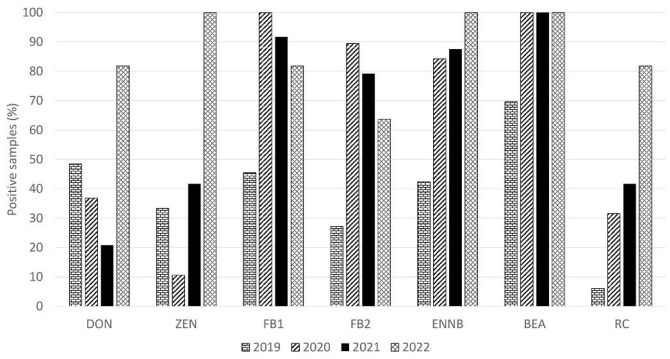

Fig. 1. Percentage of contamination across various sampling years.

Specifically, the only mycotoxin with established maximum limit is AFB_1, with a maximum tolerable level of 20 μg/kg in feed materials, which is approximately 20 times the LOQ of the method (European Commission, 2011). While other methods with lower LOQs have been previously developed (Table 4), they only allow the detection of a specific group of toxins, mainly aflatoxins (Álvarez-Días et al., 2022; Gallo et al., 2020; Xu et al., 2021), whereas the proposed method provides a comprehensive assessment of both regulated and emerging toxins.

The matrix effect arises from co-eluting compounds with the target analyte, potentially leading to the suppression or enhancement of the analytical signal. Several approaches can be employed to correct the matrix effect; in this case, the SSE factor was determined by comparing the slope of calibration curves constructed in the solvent with those in the matrix (Fabregat-Cabello et al., 2016). In general, the matrix caused signal variations lower than 25%; however, hydrophilic trichothecenes, specifically type A trichothecenes (DON and DOM-1), constitute the group of compounds most significantly affected. These toxins usually present a high SSE factor since polar compounds are generally more prone to high suppression (Lago et al., 2021). Consequently, the extraction involving the analysis of a diluted sample, while leading to relatively high detection limits, facilitated minimal matrix effect. This is particularly significant in the analysis of TMR, given the variations in composition observed from one farm to another, which can lead to important variation in the SSE caused by the matrix. Therefore, the employed approach guarantees accurate and robust results, addressing potential challenges linked to the matrix effect.

Lastly, an assessment of the accuracy was conducted. To do this, a solvent-based calibration curve was used to calculate the apparent recovery (R_A), which was then corrected with the SSE factor to obtain the R_E, which served as an accuracy measure. Simultaneously, the RSD of the recovery was used to evaluate precision. Apart from CTN, the recoveries ranged from 61% to 94%, with precision consistently below 17% (Table 1). Consequently, the method aligns with European regulations for most compounds since it meets performance criteria as outlined in the Commission Implementing Regulation (EC) 2023/2782 (European Commission, 2023). However, the method was only in-house validated, using a single concentration of toxin established at a level lower than the maximum amount allowed or recommended in feed. If the method was to be used for official analysis, it would be necessary to establish the performance characteristics with a higher number of samples and using different concentrations of toxin (European Commission, 2023). In this sense, the latest SANTE guidances considers the LOQ of the method as the lowest level that has been validated by applying the complete analytical method (SANTE, 2021).

4.2. TMR analysis

The analysis method was validated for 29 mycotoxins, and 16 of them were detected in the samples collected over the 4 years of the study (Table 3). However, the occurrence varied significantly, as it was also

Table 3
Contamination levels of the studied samples by year and season.

	Year	N	AFB_1	DON	ZEN	FB_1	FB_2	OTA	ENNA	$ENNA_1$	ENNB	$ENNB_1$	BEA	FA	AME	RC	STG	MPA
Autumn	**2019**	**33**	2.79 (1)	239 (16)	61 (11)	229 (15)	146 (9)	<LOD	<LOQ	<LOQ	108.8 (14)	133.3 (3)	220 (23)	139 (15)	<LOD	57 (2)	21 (1)	<LOD
	2020	**3**	<LOD	610 (2)	104 (1)	194 (3)	109 (2)	<LOD	7.4 (1)	11.5 (1)	12.9 (2)	10.8 (2)	38 (3)	<LOD	<LOD	<LOD	<LOD	<LOD
	2022	**2**	<LOD	547 (2)	91 (2)	<LOD	<LOD	<LOD	<LOQ	3.7 (2)	35.7 (2)	9.8 (2)	35 (2)	<LOD	<LOD	46 (1)	79 (1)	<LOD
	Total	**38**	2.79 (1)	307 (20)	69 (14)	223 (18)	139 (11)	<LOD	7.4 (1)	6.3 (3)	90.0 (18)	63.0 (7)	188 (28)	139 (15)	<LOD	54 (3)	50 (2)	<LOD
Spring	**2021**	**15**	<LOD	353 (3)	53 (8)	655 (13)	182 (10)	58 (2)	3.4 (5)	4.3 (5)	16.7 (12)	5.2 (12)	39 (15)	<LOD	22 (4)	4525 (10)	479 (5)	569 (5)
	2022	**4**	<LOD	133 (2)	198 (4)	167 (4)	52 (3)	87 (2)	0.5 (4)	2.0 (4)	31.3 (4)	11.1 (4)	7 (4)	<LOD	7 (3)	6 (4)	9 (3)	33 (1)
	Total	**19**	<LOD	265 (5)	101 (12)	540 (17)	152 (13)	73 (4)	2.1 (9)	3.3 (9)	20.4 (16)	6.6 (16)	46 (19)	<LOD	15 (7)	3234 (14)	303 (8)	480 (6)
Winter	**2020**	**16**	<LOD	443 (5)	112 (1)	214 (16)	109 (15)	36 (2)	13.1 (7)	13.6 (9)	19.6 (14)	11.9 (14)	41 (16)	153 (2)	33 (3)	96 (6)	1 (4)	<LOD
	2021	**9**	2.86 (1)	372 (2)	85 (2)	464 (9)	223 (9)	<LOD	6.9 (2)	5.7 (9)	11.4 (9)	8.4 (9)	65 (9)	<LOD	<LOD	<LOD	<LOD	<LOD
	2022	**5**	<LOD	86 (5)	244 (5)	116 (5)	48 (4)	17 (2)	3.1 (5)	2.7 (5)	5.0 (5)	4.1 (5)	12 (5)	<LOD	13 (2)	326 (4)	12 (2)	64 (2)
	Total	**30**	2.86 (1)	283 (12)	187 (8)	273 (30)	137 (28)	27 (4)	8.6 (14)	8.1 (23)	14.3 (28)	9.4 (28)	43 (30)	153 (2)	25 (5)	188 (10)	4 (6)	64 (2)

The average concentration is expressed as μg/kg, and the number of positive samples (above LOQ) is shown in brackets. Total number of samples (N).

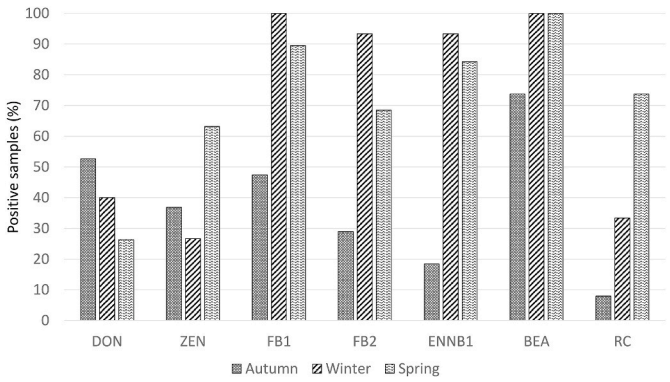

Fig. 2. Percentage of contamination based on the sampling season.

observed in previous studies (Table 4). Similar results regarding the occurrence of aflatoxins (2%) and ZEN (40%) were found in maize silage used for TMR preparation from the same geographical area. However, the occurrence of DON was higher, reaching 80%, while that of fumonisins was lower, standing at 40% (González-Jartín et al., 2022). Other study conducted in the South of Portugal detected AFB_1 in 10% of raw materials used in TMR production, while the presence of ZEN and DON was significantly higher, albeit with a limited number of samples evaluated (Viegas et al., 2020). A prior investigation assessing mycotoxins in Spanish TMR reported a prevalence of 15% for AFB_1, 17% for DON, 16% for ZEN and 34% for fumonisins. The elevated occurrence of aflatoxins may be explained by the high sensitivity exhibited by the UHPLC-FLD method and the fact that the sampling was done in farms that had presented problems related to aflatoxins in previous years (Rodríguez--Blanco, Marín, et al., 2020; Rodríguez-Blanco, Ramos, et al., 2020). Therefore, the results obtained are in the range of other studies carried out in the same geographical area while it is lower than in other countries such as Mexico or China (Álvarez-Días et al., 2022; Xu et al., 2021).

Regarding emerging compounds, the prevalence of toxins produced by *Fusarium* species, such as BEA and enniatins, is remarkable, being present in up to 88% of the samples. On the contrary, compounds produce by *Aspergillus* or *Penicillium* were less frequently found, with MPA detected in 9%, STG in 18%, and RC in 31% of samples. The only *Alternaria* toxin detected was AME, present in 14% of the samples. No prior information regarding emerging toxins in TMR from areas close to the current study location has been identified. A previous study, carried out in Mexico, detected enniatins in a similar proportion as that observed in Portugal, while AME and MPA were more frequently detected in up to 42% of TMR samples (Penagos-Tabares , Sulyok et al., 2023). Similarly, a study conducted in Pakistan detected, among others, BEA in all samples, STG in 37% and AME in 90% (Penagos-Tabares , Mahmood et al., 2023).

The seasonal disparities in sample collection may have contributed to the observed variations in toxin prevalence (Table 3). Specifically, the low prevalence of fumonisins, ENNB, and BEA in 2019 could be attributed to the fact that all 33 samples collected during that year were obtained in the autumn. Regarding DON, a notable reduction was observed in 2021, where 15 of the 24 samples were obtained during the spring. Additionally, the lowest occurrence of ZEN was noted in 2020, with 16 out of the 19 samples collected during winter. However, samples were taken most consistently during this season with other 9 samples in 2021 and 5 in 2020. Across the 4 years, the frequency of ZEN in winter was lower than in autumn and spring. On the other hand, during spring, a notable increase in samples contaminated with ZEN and RC is observed. Although the variation in toxin occurrence and concentration depending on the time of year has been previously demonstrated (González-Jartín et al., 2022), in the case of the TMR it is very difficult to predict since the origin of the raw materials used for their production can also vary.

The only toxin regulated for animal feeding is AFB_1. It was found in very few samples, with a maximum concentration of 2.9 μg/kg (Table 2), below the 5 μg/kg permitted in feed for dairy cattle (European Commission, 2011). Except for one sample that had 817 μg/kg of ZEN, exceeding the 500 μg/kg set as maximum recommended amount for dairy cattle, the observed levels for regulated mycotoxins comply with

Table 4
Summary of reports of mycotoxins in TMR and related products.

Data		Current study	González-Jartín et al. (2022)	Viegas et al. (2020)	Rodríguez-Blanco, Marín, et al. (2020), Rodríguez-Blanco, Ramos, et al. (2020)	Penagos-Tabares, Mahmood et al. (2023)	Penagos-Tabares, Sulyo et al. (2023)	Á lvarez-Días et al. (2022)	Xu et al. (2021)
Extraction and analysis, number of toxins included in the method		QuEChERS UHPLC-MS/MS, 29 mycotoxins	QuEChERS UHPLC-MS/MS, 39 mycotoxins	Solid-liquid using a SPE cartridge UHPLC-MS/MS, 16 mycotoxins	Solid-liquid, UHPLC-FLD and UHPLC-MS/MS, 9 + 5 mycotoxins	Solid-liquid, UHPLC-MS/MS, >500 mycotoxins	Solid-liquid, UHPLC-MS/MS, >800 mycotoxins and other metabolites	Solid-liquid LC-FLD, 4 toxins	Solid-liquid LC-FLD, 1 toxin
Matrix and number of samples		TMR, 87	Maize silage, 45	TMR raw materials, 9	TMR, 193	TMR, 30	TMR, 30	TMR, 99	TMR, 22
Geographical area, and season		North of Portugal, all	North of Portugal, all	South of Portugal, unknown	North of Spain, all	Pakistan, unknown	Mexico, summer	Mexico, unknown	China Autumn -Winter
Occurrence % and LOD of the detection method	**AFB_1**	2.3% (0.27 μg/kg)	2% (0.71 μg/kg)	10% (0.06 μg/kg)	15% (0.1 μg/kg)	40% (Unknown)	0% (Unknown)	100% (2.5 μg/kg)	100% (0.03 μg/kg)
	DON	42.5% (21 μg/kg)	82% (28.94 μg/kg)	80% (1 μg/kg)	17% (0.75 μg/kg)	0% (Unknown)	53% (3.6 μg/kg)	–	–
	ZEN	39.1% (7.1 μg/kg)	39% (8.34 μg/kg)	100% (0.07 μg/kg)	16% (0.25 μg/kg)	43% (Unknown)	68% (2.8 μg/kg)	–	–
	FB_1	74.7% (21 μg/kg)	41% (18.7 μg/kg)	30% (0.5 μg/kg)	34% (30 μg/kg)	93% (Unknown)	47% (16 μg/kg)	–	–
	OTA	9.2% (10.3 μg/kg)	0% (11.8 μg/kg)	50% (0.13 μg/kg)	–	7% (Unknown)	0% (Unknown)	–	–
	ENNB	71.3% (1.5 μg/kg)	50% (0.93 μg/kg)	–	–	20% (Unknown)	68% (0.9 μg/kg)	–	–
	BEA	88.5% (2.2 μg/kg)	94% (0.43 μg/kg)	–	–	100% (Unknown)	100% (0.3 μg/kg)	–	–
	RC	31% (0.5 μg/kg)	18% (7.15 μg/kg)	–	–	–	–	–	–

the recommendations (European Commission, 2006).

4.3. Carry-over to milk

To assess whether the presence of toxins in the TMR poses a direct risk for their occurrence in milk, several farms were chosen. Milk samples were collected from the milk tank, which contained the combined production of several cows, 24 h after the animals were fed with the TMR. These samples were then analyzed using a method that had been previously validated for the detection of 40 mycotoxins in this specific matrix (González-Jartín, Rodríguez-Cañás et al., 2021). No transfer was observed for toxins commonly found in TMR such as DON, ZEN and FB_1. It was previously reported that rumen microorganisms are capable of degrading these contaminants in a high proportion, while reports on FB_1 degradation are inconsistent although it seems to be barely metabolized (Loh et al., 2020). In orally exposed ruminants, fumonisins exhibit very limited bioavailability, undergoing extensive biotransformation and rapid excretion primarily through the fecal route, which may explain their absence in milk (EFSA, 2018). Moreover, no transference was neither observed for MPA, STG, or AME. MPA is an immunosuppressive agent whose pharmacokinetics has been extensively studied in humans. This small molecule exhibits high protein binding, which limits the amount of free compound available for transfer into breast milk. It can only be detected in breast milk after the administration of high doses, with minimal excretion through this route (<0.02%) (Krutsch et al., 2023). Regarding STG, the limited available information suggests that its absorption is minimal, with excretion occurring via bile and urine (EFSA, 2013). Similarly, a low absorption has been observed for AME (Islam et al., 2023).

On the contrary, a carry-over was established for RC, BEA and enniatins. The calculation method, described by Guo et al. (Guo et al., 2021) was employed, using an average intake of 43 kg of TMR. This TMR includes 30–35 kg of silage, 1.5 kg of straw, and 8–10 kg of mixture. Additionally, a daily milk production rate of 40 L per cow was considered in the analysis. In the case of RC, a transfer rate of 2.8% was obtained while for BEA, ENNA, $ENNA_1$, ENNB, and $ENNB_1$ were 1.06%, 4.85%, 6.66%, 10.10%, and 5.54%, respectively. At the moment, most research efforts are focused on studying the carry-over of aflatoxins, with reported values ranging from 1% to 6.2%. In addition, it has also been reported the transference of T-2 toxin and FB_1 with rates lower than 2 and 0.05%, respectively (Fink-Gremmels, 2008; Guo et al., 2021).

RC and enniatins are only partially degraded by rumen microbiota, making systemic absorption feasible (Debevere et al., 2020). Although enniatins and BEA have been previously found in human and animal milk, the transfer ratio has not been studied (González-Jartín, Rodríguez-Cañás et al., 2021; Pietruszka et al., 2023; Rubert et al., 2014). These compounds are *in vitro* substrates of the ABCG2 transporter, which facilitates the excretion of both endogenous and exogenous substances from the bloodstream into milk (García-Lino et al., 2019). Consequently, substrates of this transporter can appear in high concentrations in milk (Fink-Gremmels, 2008). The specific carry-over for BEA and enniatins, which are within the range of the observed one for aflatoxins, was determined for the first time in this study.

5. Conclusion

The proposed analytical method, based on a QuEChERS extraction followed by UHPLC-MS/MS detection, enables the quantification of both regulated and emerging toxins, aligning with current legislation. The study revealed a high prevalence of fumonisins and BEA, while toxins such as DON, ZEN, and RC were present in approximately 35% of the samples. On the other hand, OTA and MPA exhibited a lower occurrence, each found in less than 10% of the samples. Notably, aflatoxins were detected in only 2% of the samples, highlighting a relatively low incidence. Furthermore, the study observed variable transfer rates of enniatins, BEA, and RC from TMR to milk.

CRediT authorship contribution statement

Jesús M. González-Jartín: Writing – original draft, Validation, Investigation. **Inés Rodríguez-Cañás:** Validation, Investigation. **Rebeca Alvariño:** Validation, Investigation. **Amparo Alfonso:** Writing – review & editing, Project administration, Methodology. **María J. Sainz:** Methodology. **Mercedes R. Vieytes:** Methodology. **Ana Gomes:** Resources. **Isabel Ramos:** Resources. **Luis M. Botana:** Supervision, Funding acquisition.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2024.110682.

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