Occurrence of aflatoxin B_1 in dairy cow's feed over 10 years in Portugal (1995-2004)

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Summary

In Portugal, there is rather little information about the natural occurrence of aflatoxin in feedstuffs. The aim of this work was to report the results of screening the natural incidence of aflatoxin B_1 (AFB_1) in samples of cattle feed collected from seven dairy cow's farms from Portugal distributed in several locations of the country. One thousand and one samples were taken from 1995 to 2004. High performance liquid chromatography (HPLC) was used for separation, identification and quantification of the compound. Detection limit was 1 µg /kg. Aflatoxin B_1 was detected in 374 (37.4%) of the samples.
The incidence and mean content of AFB_1 was generally low. Levels of aflatoxin B_1 above the maximum limit established in Portugal (5 µg/kg) for dairy cattle feed samples were observed in 62 samples (6.2%) with levels ranging from 5.1 to 74 µg/kg. Out of those 62 samples, 3.7% had levels between 5.1 to 10 (mean 7.8); 1.8% had a contamination level of 10.1 to 20 (mean 12.0), and 0.7% exceeded 20.1 µg/kg (mean 50.4). On the last two years (2003-04) none of the samples exceeded the maximum permissible level of the toxin.

Key words Aflatoxin B1, Dairy cattle feed, Occurrence

Presencia de aflatoxina B_1 en piensos para ganado lechero en Portugal durante el periodo 1995-2004

Resumen

En Portugal es muy escasa la información sobre la presencia natural de aflatoxina en piensos para animales. El objetivo de este trabajo es la divulgación del estudio sobre la incidencia natural de aflatoxina B_1 (AFB_1) en muestras de piensos, recogidas en siete explotaciones ganaderas de bovinos lecheros distribuidas por diversas regiones de Portugal. Se recogieron y analizaron 1.001 muestras entre el periodo de 1995 a 2004. En la separación, identificación y cuantificación del compuesto, se utilizó la técnica de cromatografía líquida de alta resolución (HPLC). El límite de detección fue de 1 µg/kg. La aflatoxina B_1 fue detectada en 374 (37,4%) de las muestras.
La incidencia y el contenido medio de AFB_1 fueron en general, bajos.
En 62 muestras (6,2%) se observaron niveles de aflatoxina B_1 por encima del limite máximo permitido en Portugal (5 µg/kg), con concentraciones entre 5,1 y 74 µg/kg. De las 62 muestras, 3,7% presentaron concentraciones entre 5,1 y 10 µg/kg (mediana 7,8); 1,8% tenía una concentración entre 10,1 y 20 µg/kg (mediana 12,0), y 0,7% era superior a 20,1 µg/kg (mediana 50,4). En los últimos 2 años del estudio (2003-04) ninguna muestra excedió el límite máximo permitido para la toxina.

Palabras clave Aflatoxinas, Ganado lechero, Presencia, Piensos

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Mycotoxins in feedstuffs are recognised as a public health problem of considerable importance. Fungal toxins in feed and food have become a major research area since the 1961 discovery of the carcinogenicity of the aflatoxins. Fungal infestation and subsequent mycotoxins production can occur during plant growth, maturity, harvesting, processing of grains and is influenced by various factors (temperature, relative humidity, oxygen availability, damaged or broken grain kernels) [1,9].

Aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, AFM_2), a group of potent carcinogenic and teratogenic compounds, are secondary metabolic products of some *Aspergillus* spp.: *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius* [8¸10].

Since the discovery of the aflatoxins, a comprehensive literature on many aspects of their natural occurrence, chemistry and toxicology has been described. AFB_1 may occur in a wide variety of agricultural commodities such as: barley, beans, corn, cottonseed, rice, wheat and many other items like peanuts. Aflatoxins contain a coumarin nucleus fused to a bifuran and either a pentanone (AFB_1, AFB_2) or a six-membered lactone (AFG_1, AFG_2) [2,6]. AFB_1 presents the highest degree of toxicity for animals, followed by aflatoxins M_1 (AFM_1), AFG_1, AFB_2 and AFG_2 [4].

AFB_1 can increase the incidence of disease and reduce production efficiency. Some of the gross effects of AFB_1 can include: intake reduction or feed refusal, reduction in nutrient absorption and metabolism, digestive disorders including haemorrhage and necrosis. Toxicity occurs at the cellular level. AFB_1 causes DNA changes, cell deregulation, cellular changes and death [20].

When AFB_1, the mayor toxic member of the group, contaminates the feed of lactating animal, the milk from these animals will contain aflatoxin M_1 (AFM_1), the principal metabolite arising from biotransformation of AFB_1. The presence of AFM_1 in milk can be a potential threat to the health of consumers of dairy products [5].

To reduce the risk of exposure, many countries have regulated the levels of AFB_1 in feed (and have set or proposed maximum permissible levels of AFM_1 in milk). Currently, the legal limits of AFB_1 in feedstuffs are highly variable from the European Union (EU) Countries to other countries (the EU has a limit of 5 µg/kg for dairy feed) [15].

The purpose of this study is to give the information of the occurrence of AFB_1 in dairy feed over ten years of observation.

Sampling. A total of 33, 29, 25, 15, 145, 151, 139, 109, 180 and 175 complete dairy cow's feed samples, from seven dairy cow's farms from Portugal, were collected from 1995 to 2004, respectively. Samples were ground in a laboratory mill to pass a 1.0 mm, screened and mixed accurately to ensure homogeneity. If not analysed immediately, samples were stored at -20 °C. The sample size was approximately 1 kg and two sub samples of 500 g each were analysed.

Apparatus. HPLC analysis was performed by an Interface D-7000 LaChrom, Merck Hitachi; FL Detector L-7480, Merck Hitachi; Merck Column Oven L-7350 LaChrom; Pump L-7100 LaChrom, Merck Hitachi; Pump L-7110 LaChrom, Merck Hitachi. Data acquisition was performed by Compaq Deskppro software.

Determination of natural occurrence of AFB_1 by HPLC. AFB_1 was determined according to the official EC method (Commission of the European Communities, 1992; Nº L327/54) [14]. Briefly, a 50-g sample was extracted with a solvent mixture of chloroform stabilised with 0.5% of ethanol (250:25) by shaking for 30 min; after filtration through a folded filter-paper, an aliquot (50 ml) of the filtrate was passed through a Sep-Pak Florisil mini-column (Waters, Milford, MA, USA) previously conditioned with 10 ml chloroform. The column was rinsed with 10 ml chloroform followed by 20 ml methanol. AFB_1 was eluted with solvent mixture of water and acetone (85+15) and finally the purified sample extract was injected in the HPLC.

Standard AFB_1 was purchased from Sigma-Aldrich (Ref. A-6636, Química S.A. Spain). The stock solution, working standard and the calibration curve were prepared and determined as described in the Official Journal of the European Communities (1992; Nº L327/54) [14]. Determination of AFB_1 level in samples extracts was carried out by isocratic reverse-phase liquid chromatography (HPLC) using a LiChrospher 100 RP-18 (5 mm column 25 x 4.6 mm i.d.) EcoPack (Merck, Portugal), with post column derivatisation involving bromination, with pyridinum hydrobromide perbromide (PBPB) (Sigma P-3179) (Quimica S. A., Spain) and with fluorescence detector and computing integrator Merck Hitachi (Compaq Deskpro); excitation and emission wavelengths of l were 360 nm and 420 nm. The mobile phase was water-acetonitrile-methanol solution (6+2+3, v/v/v), and the flow rates were 1.00 ml/min for mobile phase and 0.30 ml/min for reagent PBPB. The retention time for the AFB_1 was 13.35 min.

Table 1. Concentration and frequency of occurrence of AFB1 in dairy feedstuffs over 10 years in Portugal

		Concentration (µg/kg)											
Years	N^+/N (%)	1-5.0			5.1-10			10.1-20			20.1-80		
		N^+	%	Mean	N^+	%	Mean	N^+	%	Mean	N^+	%	Mean
1995	7/33 (21.2)	3	9.1	1.6	4	12.1	9.5	0	0	–	0	0	–
1996	11/29 (38)	10	34.5	2	1	3.5	8	0	0	–	0	0	–
1997	8/25 (32)	4	16	1.2	0	0	0	1	4	19	3	12	35.6
1998	8/15 (36.6)	4	26.7	3	3	20	6	0	0	–	1	6.7	74
1999	97/145 (23.1)	61	42	3.5	23	15.9	7.6	10	6.9	13.3	3	2.1	41.7
2000	76/151 (50.3)	68	45	2.6	5	3.3	7	3	2	3.4	0	0	–
2001	50/139 (36)	48	34.6	2.2	1	0.7	9	1	0.7	13	0	0	–
2002	13/109 (12)	10	9.2	2.1	0	0	–	3	2.8	11.4	0	0	–
2003	78/180 (43.3)	78	43.3	2.4	0	0	–	0	0	–	0	0	–
2004	26/175 (14.9)	26	14.9	1.8	0	0	–	0	0	–	0	0	–
Total	374/1001 (37.4)	312	31.2	2.2	37	3.7	7.8	18	1.8	12.0	7	0.7	50.4

N: number of samples; N^+: positive sample; samples < 1 µg/kg were considered negatives.

The recoveries were done in duplicate, in blank dairy cattle feed samples of (1 kg), spiked with level of 4.0 mg/kg of AFB_1. The average recoveries was 98.0% Confirmation of AFB_1 identity was performed by thin layer chromatography (TLC), according to the method prescribed by Przybylski [18] and by Gimeno [3]. The identification of AFB_1 is based on derivate formation of AFB_{2a} by reaction with trifluoracetic acid (TFA) (Sigma T-6508), directly on the TLC plate SIL G-25 HR (Macherey-Nagel, ref. 809033, Germany). Samples with AFB_1 levels below 1 mg/kg were considered as negative (less than the detection limit).

The results concerning to the 2 determinations of each sample are summarised in Table 1. Three hundred and seventy four samples out of 1001 (37.4%) analysed samples for the presence of AFB_1, were positive.

The level of contamination for AFB_1 was generally low and ranged from 1µg/kg to 74 µg/kg. Levels of aflatoxin B_1 above the maximum limit established in Portugal (5 µg/kg) for dairy cattle feed samples were observed in 62 samples (6.2%) with levels ranging from 5.1 to 74 µg/kg. Out of those 62 samples, 3.7% had levels between 5.1 to 10 µg/kg (mean 7.8); 1.8% had a contamination level of 10.1 to 20 µg/kg (mean 12.0), and 0.7% exceeded 20.1 µg/kg (mean 50.4). On the last two years (2003-04) none of the samples exceeded the maximum permitted level of the toxin.

Similarly to the present study, in 1980, Patterson and Roberts [17] reported a survey over a period of 13 years on the presence of AFB1 in 740 samples of animal feedstuffs, including dairy feeds, where the toxin was detected in 27% of the dairy feed samples, at levels of more than 30 µg/kg. Oswieler and Trampel [16] found AFB1 in the same type of samples in concentrations from 0.096 to 1.700 mg/kg. Skrinjar et al. [19] detected this toxin in dairy feed samples at levels ranging from 0.005 to 0.016 mg/kg.

According to Huni et al. [7] in Swiss commercial dairy feeds the AFB1 content decreased sharply from 47 µg/kg in the winter of 1976-77 to 24 µg/Kg in the winter of 1977-78 and to 3 µg/Kg in the winter of 1978-79 and this tendency was associated to the restrictive Federal measures adopted in this country. Similarly, the present study shows that from the year 2000, there is a decrease on the concentration of the toxin found in the dairy feed samples and since 2003 none exceeded the maximum permitted level of the toxin. This tendency is probably related to increasing surveillance measures on the sanitary quality of raw materials and the subsequent feedstuffs observed in Portugal in the last years [11].

Although there are some reports on the presence of moulds, and particularly *A. flavus* in feedstuffs in Portugal [12,13] the occurrence of the toxin produced by this fungus is scarcely referred. In 2001 Martins and Martins [13], observed the contamination of moulds (*A. flavus*) in 27% of 155 bovine feed samples during the years 1996 to 1999. Albeit these data might indicate problems in the quality of the feed, correlation with the presence of AFB_1 is not possible to establish as not all the *A. flavus* strains are toxinogenic [10].

Based in the results obtained in this study, a permanent monitoring for AFB_1 by the feed industry, as well as awareness on the part of breeders is highly recommended and beneficial, since this mycotoxin affects animal's health and the contaminated milk can be a serious public health hazard.

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