Screening of Aflatoxin B_1 in Laboratory Rat Feed

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Summary

Many hazards can interfere with the safety of the feeding stuffs intended to provide nutrients to experimental animals. Chemical and biological contaminants of laboratory animal feed can be a problem for toxicological and immunological research. Aflatoxin B_1 (AFB_1) is a secondary toxic metabolite, produced by the ubiquitous fungal genera, *Aspergillu*s. AFB_1 is particularly dangerous for health, inducing cancer of the urinary tract or liver carcinoma. The aim of this preliminary screening was to evaluate the presence of AFB_1 in 31 samples of laboratory rat feed using a method validated in-house, then separated by High Pressure Liquid Chromatography (HPLC) coupled to a fluorescence detector. The detection limit (limit of detection: LOD) and the quantification limit (LOQ) were 0.2 and 0.4 µg/kg respectively. Recoveries ranged from 58.0 to 74.5% for spiked samples. The immunoaffinity approach was significantly faster than methods employing conventional chromatography clean-up (Sep-Pak Classic Florisil and Sep-Pak ClassicC_{18} cartridges). Aflatoxins were not detected in any analysed sample.

Introduction

Laboratory animals are fed either for life maintenance or for testing some particular effect of specific nutrients in a test diet (*Beynen & Coats, 2001*). The health status, performance and metabolism of experimental animals are influenced by the composition of the diet and the feeding practice. In all situations, safety of the feed is an essential parameter for the consistency of the results obtained with those experimental animals. Many hazards can interfere with the safe use of the feeding stuffs intended to provide nutrients to experimental animals: biological or chemical ones are the most relevant.

Cereals and seed protein are the ingredients generally used to obtain the final feed. Unfortunately this type of material has been associated with contaminants produced by moulds during stages of the crop production. Among crop contaminants, aflatoxins, produced by moulds, are the most prevalent and hazardous.

Moulds are widely distributed in the environment. Mould contamination not only causes deterioration of food and feeds, but also can adversely affect the health of humans and animals, since they may produce toxic metabolites - mycotoxins (*Joseph, 1971; Cole & Cox, 1981*).

The three major economically important genera of mycotoxin-producing fungi are *Aspergillus, Penicillium* and *Fusarium.* They are ubiquitous, can cause food spoilage and biodeterioration, and are capable of producing many different mycotoxins (*Thomas et al., 1987; Cawood et al., 1991*). *Aspergillus flavus*, *Aspergillus parasiticus* and *A. nomiu*s (*Kurtzman et al., 1987; Betina, 1989*) are widespread fungi isolated from a wide range of animal and human foods, which produce highly hepatocarcinogenic compounds, the aflatoxins. Since

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the 1961 discovery of the carcinogenicity of these toxins (*Joseph, 1971*), fungal toxins in raw material, feed and foods has become a major research area. Aflatoxins are considered at present to be one of the most dangerous contaminants in raw material, feed and food. One of most characteristic features of aflatoxins is their high toxicity and carcinogenic potency for a large number of animal species (*Cole & Cox, 1981*).

Aflatoxin B_1 (AFB_1) is classified in the difurocoumarocyclopentenone series and the difurocoumarolactones. The toxin is metabolically converted to a variety of stable metabolites (aflatoxicol, aflatoxin M_1 and aflatoxin Q_1) "in vivo" or "in vitro" in the presence of a NADPH - generating system and cytochrome P-450, which is a requisite step in the formation of the putative active biological effects: carcinogenicity, DNA binding, cytotoxicity and bacterial mutagenicity (*Coulombe et al., 1984*).

Many reports describe the contamination of animal feed by AFB_1. However, few studies have been performed in feed produced for experimental animals and in Portugal there is no information about the presence of AFB_1 in this material. The aim of this study was to make a preliminary screening of aflatoxin B_1 in laboratory rat feed by High Performance Liquid Chromatography using an immunoaffinity column clean-up and also to assess the validity of this procedure the actual determination of AFB_1 in feed.

Materials and Methods

Samples collection

A total of thirty one pelleted samples destined for laboratory rats were collected from two research laboratories in the Lisbon area (Portugal). No particular preference was used in selecting samples or locations. All samples were transported and stored at room temperature (20ºC).

Declared ingredients from pelleted rat feed were: wheat (50.0%), soy (13.8%), barley (13.0%), maize (MGO - organism genetically modified), sunflower seed and fat; the nutritional composition was: total protein (15.4%), cellulose (4.1%), ash (5.9%) and additives: vitamins A, D3, E (alfa tocoferol), copper and copper sulphate.

Standard solutions

Standard AFB_1 (A-6636) was obtained from Sigma-Aldrich (Spain). Working standard solutions were prepared in toluene/acetonitrile (98+2 by volume) (10 mg/ml). AFB_1 stock solutions were determined by measuring the absorbance at 363nm. These solutions were stored below –18ºC in the dark.

Immunoaffinity columns (IAC)

The immunoaffinty columns Afla B (G.1003) were provide by VICAM (Watertown, USA).

Chemicals

All reagents and solvents of analytical grade purity were provided by VWR (Darmstadt, Germany).

Extraction/purification method for AFB_1

AFB_1 analysis was carried out following the method described by ISO/FDIS 17375:2006.

The samples were analysed for AFs using immunoaffinity columns supplied from VICAM (USA), and quantified by high pressure liquid chromatography (HPLC). The quantification limit was 0.4µg/kg.

In detail, a 50g sample was extracted with a solvent mixture of acetone/water (85+15, v/v). The sample extract was filtered, diluted and applied to an immunoaffinity column containing antibodies specific to aflatoxins B_1. The Aflatoxin B_1 fraction was eluted with methanol.

Determination of aflatoxin B_1 levels in sample extracts was carried out by isocratic reverse-phase liquid chromatography (HPLC) using a LiChrospher 100 RP-18 5 µm column (25 x 4.6 mm i.d.) EcoPack (Merck, Portugal), with post-column derivatisation involving bromination with pyridinium hydrobromide perbromide (PBPB) (Sigma P- 3179) (Quimica S. A., Spain) and with a fluorescence detector and computing integrator Merck Hitachi (Compaq Deskpro); excitation and emission wavelengths of λ 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 11.11 min

Detection limit (LOD) and percentage recovery were determined according to the method previously referred. The limit of detection was 0.2 mg/kg. Recovery was determined by spiking AFB_1 standards at levels of 0.4-2.0 mg/kg to samples. Recovery ranged from 58.0-74.5 %.

AFB_1 recoveries

The recoveries were done in duplicate, in blank samples (500g). The recovery ranged from 58.0-74.5 % (Table 1). Five feeding stuffs samples were spiked with 0.4, 0.5, 0.6, 1.0 and 2.0 mg/kg and analyzed by the same operator on the same day with the same HPLC system.

Calibration curve for AFB_1 analysis

Table 1. Results of recoveries of AFB_1

C- AFB_1 concentration

C (µg/kg)	Recovery (%)
0.40	69.9
0.50	74.5
0.60	58.0
1.00	73.9
2.00	70.8

Legend: C- AFB_1 concentration

The stock solution, working standards and the calibration curve were prepared and determined as described by ISO/ FDIS 17375:2006. Four standard solutions with a quantity of AFB_1 equivalent to a contamination of 1.75 to 5.50 µg/kg were analysed by HPLC. The coefficient of linearity (R^2) was 0.9998 (Figure 1). The LOD was 0.2µg/kg and the LOQ was 0.4µg/kg.

Repeatability test for AFB_1 analysis

Two samples were spiked with 1.75mg/kg and 5.5mg/kg and analysed ten times by the same operator on the same day with the same HPLC system (Table 2).

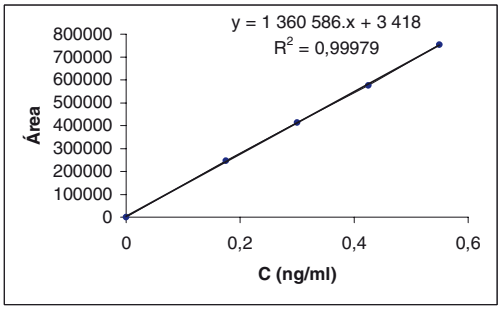

Figure 1. Calibration Curve for AFB_1 analysis
C – AFB_1 concentration

Table 2. Results of repeatability (r) and coefficients of variation (CV_r) for AFB_1

AFB_1 concentration

C (µg/kg)	r	CV_r (%)
1.75	0.015	3.1
5.50	0.024	1.5

Legend: AFB_1 concentration

Results and Discussion

The method used in this study was simple and proved to be suitable for analysing AFB_1 in feed because there is a consensus between the results obtained and the limits established in the *Official Journal of the European Communities L 201/100, 17/7/98*, Reg.1525/98. The results showed none of the samples to be contaminated with aflatoxins. This study complements some previous studies in Portugal on mycotoxin occurrence in raw materials and feed (*Martins & Martins, 2000, 2001a, b; Martins, 2003; Guerra et al., 2005; Martins et al., 2006*). On the other hand reports of the occurrence of aflatoxin B_1 in laboratory rat feeding stuffs do not exist.

In Sweden, Waldermarson et al. (2005) found naturally occurring mycotoxins in mixed feed samples for laboratory rodents. These authors detected levels of deoxynivalenol up to 298 µg/kg, nivalenol up

to 118µg/kg, ochratoxin A up to 3.1µg/kg and zearalenone up to 26.4 µg/kg. Toxins T-2 and Ht-2 were not detected in any of the samples neither aflatoxin, which is in accordance with the present study.

The recovery of AFB_1 greatly depends on chosen clean-up solvents and on the types of feeding stuffs used: the HPLC analysis which followed could be impaired by the presence of various contaminants (*Dragacci et al, 1995*). Mortimer *et al. (1987)* reported that the use of immunoaffinity gives significantly cleaner extracts, which improves the accuracy and precision of the assays, particularly when the expected levels of AFB_1 are low.

Although in the present study no AFB1 was detected, the authors suggest a permanent monitoring for AFB_1 and others mycotoxins by the feed industry, as well as awareness on the part of breeders, since this mycotoxin affects animal's health.

Control measures should hence be followed. In fact, the European legislation prohibits the mixture of different batches of raw materials contaminated and non-contaminated in order to dilute, and consequently reduce, the contamination levels in the final feed product. According to EC Regulation Nº 185/2005 of 12 January, feed plants have to apply the HACCP to the processing of each feed batch and the monitoring of mycotoxins in raw material is considered as a prerequisite.

Because the crops used in production of feeds can also be contaminated by other genera moulds, it is justified to widen the aim of these kinds of studies, namely searching for ochratoxin A, vomitoxin, zearalenone, fumonisin and T2-toxin. In the near future this methodology will be widened to feed produced for other laboratory experimental animals (mice, guinea pigs, rabbits) and to other mycotoxins.

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