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AN OVERVIEW OF MYCOTOXINS AND TOXIGENIC FUNGI IN PORTUGAL

Data collected from 1999 to July 2003

1. INTRODUCTION

The fungi are cosmopolitan and ubiquitous and have a strong ecological link with vegetable products. The natural mycobiota existing in conjunction with food and feed are of interest to society, since many of these fungi can produce compounds of interest (*e.g.*, antibiotics, enzymes or organic acids). On the other hand, they may also be detrimental by promoting food and feed deterioration. Often, the mycobiota of food commodities is dominated by toxigenic species, such as *Aspergillus*, *Fusarium* and *Penicillium*, which may produce micotoxins with effects on human and animal health. Aflatoxins, ochratoxins, patulin and the *Fusarium* toxins are considered to be the most important of the mycotoxins. Losses arising from the presence of mycotoxins have a large impact on the economy of countries.
In this report, we summarised the results obtained in Portuguese institutions with responsibility for food and feed inspection, and in mycological food and feed research. The aim is to show the natural occurrence of some mycotoxins produced by species from *Aspergillus*, *Fusarium* and *Penicillium* in products of the food and feed chains. Some of these products are imported and others are produced in Portugal. Data on the occurrence of aflatoxins, ochratoxin A, patulin and some *Fusarium* toxins are reported.

2. AFLATOXINS IN FOOD

2.1. Aflatoxin B_1, B_2, G_1, G_2, and M_1

The *Direcção Geral de Fiscalização e Controlo de Qualidade Alimentar* (DGFCQA, Directorate General of Food Inspection and Quality Control) is the responsible authority for the sampling and analysis of food commodities on sale in retail stores. It is also the responsible organissation for mycotoxin control at import points. In both cases, all products exceeding the legal limits in the EU (Commission Regulation n. 466/2001) are withdrawn from shelves or rejected, and not allowed to enter the food chain, as imposed by law.

From 1999 to July 2003, several samples of dried fruits, spices and milk were analysed. Samples of dried fruits and nuts (1242), 83 samples from difference spices, and 69 samples of milk were collected in stores and at import control points for aflatoxins analysis (Tables 1, 2 and 3, respectively).

Table 1. Occurrence of aflatoxins in dried fruits and nuts from 1999 to July 2003 (number of samples with each aflatoxin in brackets)

Product	*Number of samples*	*Positive samples (%)*	*Aflatoxins content Min. – Max. (µg/kg)*			
			B_1	*B_2*	*G_1*	*G_2*
Almonds	56	14	0.3 – 147 (8)	11.5– 20.8 (4)	0.9 – 15.5 (3)	1.6 – 2.7 (3)
Peanuts	745	18	0.4– 777.3 (132)	0.3– 125.1 (71)	0.2 – 19.2 (28)	0.2– 28.3 (11)
Hazelnuts	22	14	< 0.6 – 0.9 (3)	nd	< 0.9– 4.1 (3)	< 0.8 (2)
Cashew-nuts	23	22	< 0.3 – 0.8 (5)	0.1– 0.3 (2)	0.2 – 0.8 (2)	nd
Dry figs	303	82	0.6– 141.5 (250)	0.3 – 17.9 (127)	0.8– 172.6 (88)	0.8 – 23 (17)
Nuts	15	6	nd	nd	2.5 (1)	nd
Pine seeds	3	0	nd	nd	nd	nd
Pistacchio nuts	58	64	0.6– 323.5 (37)	0.3 – 37.2 (21)	0.3 – 3.9 (2)	< 0.4 (1)
Raisins	17	0	nd	nd	nd	nd

nd = not detected

Samples were analysed by HPLC, following the methodology recommended by CEN (European Committee for Standardization). For those commodities where specific methodology was not available, adaptations were made to existing methods. The limit of detection (LOD) for aflatoxin B_1 (AFB_1), aflatoxin B_2, aflatoxin G_1 and aflatoxin G_2 varied with the product in analyses (Table 1). For aflatoxin M_1 the LOD was 0.003µg/L.

The results indicate high levels in some dried fruits and nuts, such as almonds, peanuts, dry figs and pistachio nuts (Table 1). Aflatoxins were also observed in curry, garlic, nutmeg, and white pepper, but only in curry and nutmeg were the recommended limits for AFB_1 exceeded (5 µg/kg) (Table 2). The levels of aflatoxin M_1 in milk samples were well below the Eu limit of 0.05 µg/L (Table 3).

Table 2. Occurrence of aflatoxins in spices from 1999 to July 2003 (number of samples with each aflatoxin in brackets)

Product	*Number of samples*	*Positive samples (%)*	*Aflatoxins content Min. – Max. (µg/kg)*			
			B_1	B_2	G_1	G_2
Chilli	1	100	11.9 (1)	0.7 (1)	nd	nd
Barbecue mixture	6	66	0.2 – 2.1 (4)	0.1– 0.17 (2)	0.2 – 2.3 (2)	0.6 – 3.1 (2)
Cinnamon	1	0	nd	nd	nd	nd
Curry	11	90	0.5 –10 (10)	0.1 – 1.1 (4)	0.1 – 4.4 (3)	0.7 – 4.6 (7)
Coriander	2	50	1.9 (1)	0.17 (1)	< 0.2 (1)	nd
Cumin	1	0	nd	nd	nd	nd
Nutmeg	9	77	0.6 –10 (7)	0.4 – 1.6 (4)	0.8 – 1.5 (3)	nd
Origum	1	0	nd	nd	nd	nd
Paprika	30	90	0.1 – 3.6 (27)	0.1 – 2.4 (26)	0.2 – 4.7 (25)	2.3 (1)
Piri - piri	1	100	6.2	0.4	nd	nd
White pepper	9	100	0.4 – 0.8 (8)	0.3 (9)	1.0 – 1.2 (6)	0.5 – 1.4 (9)
Garlic	9	100	0.6 (1)	nd	nd	0.5 – 1.1 (9)
Saffron	2	50	1.5 (1)	0.26 (1)	nd	nd

nd = not detected

Table 3. Occurrence of aflatoxin M_1 in milk samples

Year	*Number of samples*	*Positive samples*	*Aflatoxin M_1 content (µg/L) min.-max.*
1999	54	51	0.010 – 0.022
2000	2	2	0.01 – 0.01
2001	12	7	<0.018 – 0.024

2.2. Aflatoxin B_1 and mycotoxigenic fungi in feeds

The National Laboratory for Veterinary Research (*Laboratório Nacional de Investigação Veterinária* - LNIV) is the driving force of mycotoxin control in feeds. Mainly bovine, poultry and swine feeds are analysed (Martins, 2003). All bovine feeds were for dairy cattle, which are responsible for the production of aflatoxin M_1 in milk for human consumption. From 1999 to 2002, 558 feed samples were analysed (Table 4) The LOD for AFB_1 detection method was 1 µg/kg. Besides the mycotoxin determination, these samples were also screened for the presence of fungi (Figures 1, 2, and 3).

Table 4. Occurrence of aflatoxin B_1 and filamentous fungi in feedstuff

Product	*number of samples*	*positive samples (%)*		*min / max (µg/kg)*
		AFB_1	*fungi*	
Bovine feed	399	34	4	5 / 15
Poultry feed	85	16	93	1 / 20
Swine feed	74	7	71	1 / 2

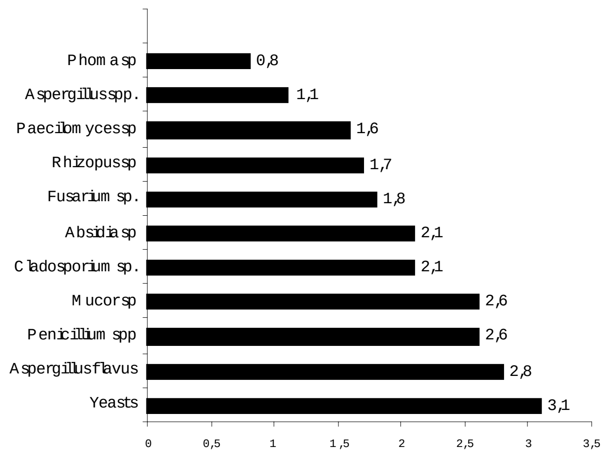

Figure 1. Means of log colony-forming units of fungal species in positive bovine feed samples (dairy cattle).

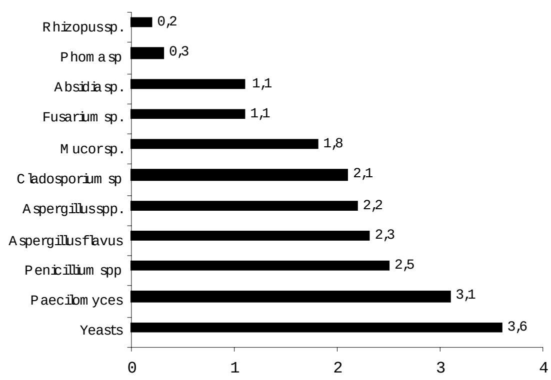

Figure 2. Means of log colony-forming units of fungal species in positive poultry feed samples.

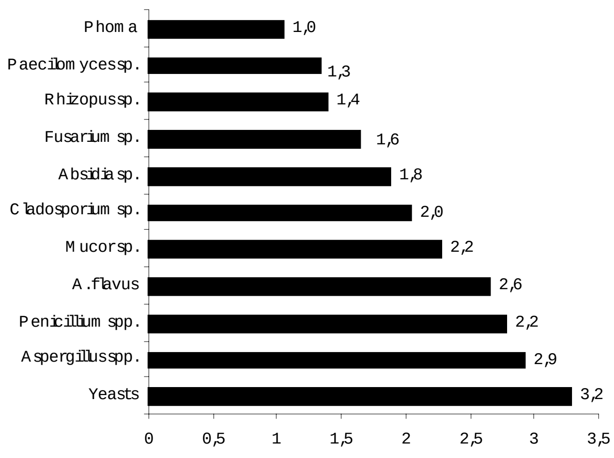

Figure 3. Means of log colony-forming units of fungal species in positive swine feed samples.

When comparing these data with those previously reported (Martins & Martins, 2001), it is concluded that there was a decrease in AFB_1 in terms of percentage positive samples and concentration. These may be the result of the new technology

employed during harvest and storage (rapid drying, correct storage of the harvested crops and use of effective anti-mould preservatives). The high percentage of bovine samples without mycological contamination may be explained by the fact that all samples were granulated. The maximum limit of aflatoxin B_1 recommended is 5μg/kg for bovine feed (dairy cattle), and 20 μg/kg for poultry and swine feed (Decreto-Lei n. 182/99). Among the 34 % of positive samples of bovine feed, just 9.5 % (13 samples) exceeded the recommended maximum limit. In the case of poultry feeds, only 2.4 % of samples (2) attained this limit.
The occurrence of AFB_1 in these feeds was correlated with the presence of *Aspergillus flavus*. As it can be seen from Figures 1 to 3, the number of strains from this species is among the predominant ones.

3. OCHRATOXIN A

From 1999 to July 2003, 410 samples were analysed for OTA in different foods and drinks (Table 5). Some of them were collected by DGFCQA in retail stores, mainly in the area of Lisbon; others arrived at the laboratory of *Departamento de Tecnologia das Indústrias Alimentares* for quality control analysis. They were analysed by HPLC according to the methodology proposed by CEN, with a LOD depending on the type of sample. Sixty-four samples of coffee were analysed by immunochemical method (RidaScreen Fast Ochratoxin A assay), being the LOD of this method 5 ppb (Lourenço *et al.*, 2002).

Table 5. Occurrence of ochratoxin A in different food commodities from 1999–July 2003 (adapted from Vicente et al., 2001; Lourenço et al., 2002;DGFCQA, personnel communication)

Product		*Number of samples*	*positive samples (%)*	*OTA level (μg/kg)*
Coffee	Green	257	89	<0.3 – 30.1
	Roasted	66	77	<1.0– 12.1
Raisins		9	89	<0.3 – 13.9
Bonnet – pepper		3	66	1.1 – 1.1
Paprika		3	100	1.1 – 4.3
Nutmeg		3	100	0.8 – 8.5
Pepper		1	0	nd
Barbecue mixture		15	86	<0.3 – 52.8
Breakfast cereals		15	60	<0.3 – 0.7
Beer	National	13	13	0.003 – 0.01
	Imported	15	60	0.002 – 0.064
Grits		5	100	0.202 – 0.204
Malt		5	100	0.121 – 0.201

Current EU legislation for OTA, includes its presence in (a) cereal and cereal products, and (b) dried vine fruits or raisins (Commission Regulation n. 472/2002). Limits for other food commodities are being considered. According to Table 5, just

some samples of raisins may exceed the EU limit, although one sample – barbecue mixture – presented a very high value of OTA – 52.8 µg/kg. Coffee samples, either green or roasted, have a high incidence of OTA. Limits for coffee may be desirable, especially because of its widespread consumption (Paterson *et al.*, 2001).
The presence of OTA in grapes and grape products – namely wine, is being assessed by different research groups in Portugal (Abrunhosa *et al.*, 2003; Alves, 2003). In the literature was found data for Port Wine, for table wine, and for grapes for wine making (Table 6). The LOD varies between 0.02 and 0.084 µg/L, and 0.03 and 0.10 µg/kg for wine and grapes, respectively. Although there are yet no limit for the presence of OTA in wine, the International Office of Vine and Wine recommends a value of 2 µg/L, from the 2005 vintage. This value was reached in only 1 from 401 wine samples (Alves, 2003).

Table 6. Occurrence of OTA in grapes, Port Wine, and table wine (from Alves, 2003; Festas et al., 2000; Serra et al., in press)

Product	*Number of samples*	*Positive samples (%)*	*LOD µg/L*	*Mean / max. µg/L*
Port Wine	189	17	0.084	0.137 / 0.474
	31	0	0.02	--
Table wine	151	24	0.084	0.179 / 2.08
	30	0	0.02	--
Grapes	58	1	0.105	0.110
	11	3	0.03	0.014 / 0.061

3.1. Ochratoxin A producing fungi in grapes

The incidence of filamentous fungi producing OTA from Portuguese wine grapes is being evaluated, by conducting surveys in vineyards, from four winemaking regions. From setting to the harvesting period, berries were sampled by plating methods for filamentous fungi isolation and identification. From the *Aspergillus* and *Penicillium* strains isolated, 14 % of the *Aspergillus* strains and none of the *Penicillium* strains were OTA producing ones (Serra *et al.*, 2002; Serra *et al.*, 2003). All *Aspergillus* strains were tested *in vitro* for OTA production, with *A. carbonarius* (97 %) and *A. niger* aggregate (4 %) strains being OTA producers. Almost all ochratoxigenic strains were isolated at harvest time (Table 7), mainly in regions with a Mediterranean climate. The vineyards from regions with Atlantic influences - Vinhos Verdes region, with high rainfall, exhibited the lowest occurrence of *Aspergillus* and ochratoxigenic strains.

Table 7. Ochratoxigenic fungi isolated from grapes at different maturation stages of grapes

Region	*Vineyard*	*% of berries with ochratoxigenic fungi*		
		Green berry	*Veraisson*	*Ripe berry (harvest)*
Vinhos Verdes	1	nd	nd	nd
	2	nd	nd	2 % *A. carbonarius*
	3	nd	nd	nd
Douro	4	nd	nd	8 % *A. carbonarius*
	5	nd	nd	20 % *A. niger* aggregate
	6	nd	nd	2 % *A. niger* aggregate
Ribatejo	7	nd	2 % *A. carbonarius*	12 % *A. carbonarius*
	8	nd	nd	2 % *A. carbonarius*
	9	nd	nd	nd
Alentejo	10	nd	nd	38 % *A. carbonarius* 2 % *A. niger* aggregate
	11	nd	nd	2 % *A. carbonarius*

nd = not detected

4. PATULIN

Data on the occurrence of patulin in 36 apple and pear samples collected in Portugal retail stores from August to October of 2001 and analysed by HPLC are presented (Table 8) (Majerus & Kapp, 2002; Novo & Felgueiras, 2002). The method of analysis follows the Portuguese recommendation, with the LOD calculated as 5µg/L.

Recent EU legislation (Commission Regulation 1425/2003) for patulin defines a limit value of 50µg/kg in fruit juices and nectar, and in based spirit drinks; a value of 25µg/kg in solid apple products; and a value of 10 µg/kg in products intended for children and young infants. Values reported in Table 7 are well below these limit values, as long as they are not intended for children and young infants. Juices with milk are mainly consumed by children; however, none of these samples contained detectable amounts of patulin.

Martins *et al.* (2002) reported the occurrence of patulin in 351 Portuguese apples with small rotten spots. Even after having used a TLC tecnique with a LOD of 120µg/kg, these authors were able to detect patulin in 68.6 % of those apples. In some of these samples the level of patulin were higher than 80mg/kg.

Table 8. Occurrence of Patulin in Apple and Pear juices (adapted from Majerus & Kapp, 2002)

Product	*Number of samples*	*% positive samples*	*µg/L-Patulin* min -max.	mean
Clear apple juice	7	-----	nd	
Cloudy apple juice (nectar)	8	88	4.0 – 25.2	10.5
Apple and other fruit juices	5	20	5.6	
Apple juice and milk	6	0	nd	
Apple puree	3	0	nd	
Cloudy pear juice (nectar)	7	29	12.6 – 23.4	18.0
Pear and other fruit juices	2	100	7.2 – 9.6	8.4
Pear juice and milk	2	0	nd	
Pear puree	2	0	nd	

nd = not detected

5. *FUSARIUM* TOXINS

The occurrence of fumonisin B_1 (FB_1) in corn and poultry feed, and in oat and horse feed was assessed due the high sensitivity of horses to this mycotoxin, and possible effects in humans. In Table 9, data on 46 samples are presented, the determination being made by HPLC with immunoaffinity clean up, and the LOD calculated as 10 ng/kg. The recommended level of FB_1 in total feed fixed by US Food and Drug Administration (FDA) is 1 mg/kg for horse feed and 50 mg/kg for poultry feed. From 46 samples, 36 were positive for FB_1 (78.3 %). The highest FB_1 content was detected on a corn sample (32.2 mg/kg). All horse feed samples had contents lower than the recommended values for FB_1. The control of mycotoxins in animal feeding stuffs is very important as they may cause reduced productivity or other symptoms. Hence, they may be transmitted to humans.

Table 9. Fumonisin B_1 content in feedstuff (from Novo et al., 2000)

Product	*Number of samples*	*positive samples (%)*	*mg FB_1/Kg* min. - max.	*mean*
Corn	12	67	0.025 – 32.20	11.911
Oat	5	40	0.132 – 0.421	0.277
Poultry feed	22	90	0.031 – 7.437	1.177
Horse feed	7	86	0.06 – 0.500	0.307

The occurrence in Portugal of three *Fusarium* toxins in foods – deoxynivalenol (DON), T2-toxin and zearalenone (ZEA) – is mentioned in the EU report, SCOOP task 3.2.10, on the occurrence of these mycotoxins (Gareis, 2003), although just a few determination were made on these toxins (Table 10).

Table 10. Occurrence of some Fusarium toxins in different food commodities (adapted from Gareis, 2003)

toxin	*Product*	*Number of samples*	*positive samples (%)*	*LOD*	*mean / max. µg/Kg*
ZEA	Wheat	1	0	2.5	nd
	White wheat flour	1	0		nd
	Wheat bran	2	100		13 / 15
	Cereals breakfast	11	64		5.1 / 11
DON	Wheat	3	33	25	256 / 744
	White wheat flour	3	33		119 / 333
	Wheat bran	4	50		761 / 1821
	Cereals breakfast	10	100		161 / 426
T2-Toxin	Wheat	9	0	37	nd
	corn	10	0		nd

nd = not detected

6. CONCLUSION

Toxigenic fungi are cosmopolitan and ubiquitous, in the agricultural environment, such as soil, cereal grain, others grains, oil seed, fruits, spices, etc... Filamentous fungi are responsible for crop losses, because of their presence, and the possible production and accumulation of mycotoxins in these food commodities.

Current legislation in Portugal comprises the presence of aflatoxins, OTA, and patulin is some food commodities, and of aflatoxins in feedstuff. Data herewith reported concern the period from January 1999 to July 2003. Probably because of this legislation, larger amounts of data are available for aflatoxins and ochratoxin A, and few data were found for patulin (legislation for patulin is very recent), and some *Fusarium* toxins.

It is worthwhile mention that some data supplied by DGFCQA concern samples collected at import control points, and not samples from food commodities on sale in local stores. This explains the high values of total aflatoxin exhibit by several samples of dried fruits and nuts (Table 1). Samples collected at retail stores had much lower levels.

OTA is the second more studied mycotoxin in Portugal by far. The incidence of OTA in food commodities seems to be high, but at low levels. Just in a few cases (roasted coffee, raisins and a barbecue mixture), OTA reaches levels of some concern.

Research efforts are ongoing on the evaluation of the presence of OTA in Portuguese wines. In this case the incidence of OTA seems to be low, and at levels far from the limits currently in discussion. In only one situation, was it possible to find a wine sample with an OTA level close to the proposed limit.
Concerning other mycotoxins (patulin and *Fusarium* toxins), the current amount of information is not enough to define trends, and more is required.
The work of Martins et al. (2002) with rotten apples emphasizes the risk of human exposure to patulin through the comsumption of fruits or fruit based foods and drinks manufactured from apples with small rotten areas.

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8. AFFILIATIONS

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