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Fusarium mycotoxins and ochratoxin A in cereals and cereal products

Results from the Bavarian Health and Food Safety Authority in 2004

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Results from the Bavarian Health and Food Safety Authority on contamination of cereals and cereal products from the Bavarian market with the mycotoxins deoxynivalenol (DON), zearalenone (ZEA), and ochratoxin A (OTA) and of maize meal and semolina with fumonisins (FUM) in the year 2004 are presented. Contamination rates and levels of DON, ZEA, and OTA were low and did not exceed the maximum levels. However, a 92% contamination rate and high levels of FUM in maize meal and semolina were measured. Contamination levels of mycotoxins are discussed and evaluated with respect to possible health implications for consumers.

Keywords: Cereals / Fumonisin / Maize / Mycotoxin / Zearalenone

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1 Introduction

The infection of cereal crops by phytopathogenic *Fusarium* fungi in the field and by fungi of the genera *Aspergillus* and *Penicillium* during processing and storage leads to entry of different secondary fungal metabolites, the mycotoxins, into the food chain. The most common mycotoxins in cereals are the *Fusarium* mycotoxins deoxynivalenol (DON), zearalenone (ZEA), and the fumonisins (FUM) and *Penicillium* and *Aspergillus* mycotoxin ochratoxin A (OTA).

Among these mycotoxins, DON, and ZEA are produced in various cereals, in particular wheat and maize, while FUM is predominately formed in maize. For DON, which belongs to the class of trichothecenes, the general toxicity and immunotoxicity are considered to be the critical effects (Scientific Committee on Food, http://www.europa.eu.int/comm/food/fs/sc/scf/out123_en.pdf). There are no indications of carcinogenic, teratogenic, or mutagenic effects. ZEA is known as an estrogenic mycotoxin leading to fertility disorders (Scientific Committee on Food, http://www.europa.eu.int/comm/food/fs/sc/scf/out65_en.pdf).

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Abbreviations: DON, deoxynivalenol; **EC,** European Commission; **FUM,** fumonisins; **OTA,** ochratoxin A; **ZEA,** zearalenone

FUM has carcinogenic properties the major target organs affected are liver and kidney. Its mode of action is primarily explained by interference with the biosynthesis of sphingolipids, which results in damage of membrane structure (Scientific Committee on Food, http://www.europa.eu.int/comm/food/fs/sc/scf/out73_en.pdf). Recent research showed a teratogenic effect of FUM caused by interaction of intake of folic acid [1]. The isocoumarin derivative OTA is the main mycotoxin in the group of ochratoxins. It is a mycotoxin with carcinogenic, nephrotoxic, teratogenic, and immunotoxic properties and is produced mainly during storage. OTA is generally found in cereals, but also in a series of other products as oleaginous seeds, coffee, pulses, and wine (Scientific Committee on Food, http://europa.eu.int/comm/food/fs/scoop/3.2.7_en.pdf).

Both the *Fusarium* mycotoxins and OTA were evaluated by the Scientific Committee on Food (SCF) in a set of opinions (http://www.europa.eu.int/comm/food/fs/sc/scf/out123_en.pdf, http://www.europa.eu.int/comm/food/fs/sc/scf/out65_en.pdf, http://www.europa.eu.int/comm/food/fs/sc/scf/out73_en.pdf, http://www.europa.eu.int/comm/food/fs/sc/scf/out14_en.html). For OTA maximum levels in cereals and cereal products have been set by the European Commission (EC) in 2002 [2]. DON, ZEA, and FUM have first been regulated in Germany in 2004 [3]. In 2005 the EC set higher maximum levels for DON and ZEA, but not for FUM, which shall apply from July 2006 [4].

The present study gives a survey on the contamination levels of cereals and cereal products from the Bavarian mar-

ket with DON, ZEA, and OTA. In addition, results on FUM-contamination of maize meals and semolina analyzed in 2004 are presented and compared with that obtained in 2003 and 2005.

2 Materials and methods

2.1 Materials

Sampling of 77 samples from cereals and cereal products and 37 samples from maize meals and semolina was performed mainly at wholesale and retail stages. With one exception only cereal grains intended for direct human consumption were analyzed.

2.2 Reagents

Standards of DON, ZEA, and OTA were purchased from Sigma-Aldrich (Deisenhofen, Germany). The standards of FUM were obtained from W. C. A. Gelderblom (Tygerberg, South Africa). Purities of standards were ≥97.0% (DON) and ≥98.0% (ZEA, OTA, and FUM). Water used for HPLC was purified in a MilliQ® water purification system (Millipore, Schwalbach, Germany) and degassed, methanol and ACN were HPLC grade (Promochem, Wesel, Germany). All other chemicals used were of analytical purity.

2.3 Methods applied

Extraction and determination of FUM and OTA were performed according to the methods of § 35 LMBG (FUM: L 15.05-2, OTA: L 15.03-1) with minor modifications. ZEA and DON were analyzed as described by R-Biopharm in the instruction manuals of the respective immunoaffinity columns.

The methods were successfully validated by interlaboratory tests. Recoveries were 97% for ZEA, 85 – 97% for DON, 73 – 100% for OTA, and 94 – 107% for FUM. The LOQ achieved with these methods were 4 µg/kg for ZEA, 50 µg/kg for DON, 0.1 µg/kg for OTA, and 5 µg/kg for FUM.

2.4 Sample preparation

2.4.1 ZEA

Ground samples (25 g) were extracted with ACN/water (75 : 25 v/v) using a high-speed Ultra-Turrax homogenizer (Janke & Kunkel IKA-Labortechnik, Staufen, Germany) for 2 min. The extract was filtered through a Whatman No. 4 filter paper (Whatman, Dassel, Germany). Twenty milliliters of this extract were diluted with water to exact 100 mL in a measuring flask. For clean up 25 mL of the dilution were passed through an immunoaffinity column (EASI-EXTRACT® ZEARALENONE, R-Biopharm, Glasgow, Scotland). Then this column was washed with 20 mL of water. The toxins were obtained by elution with 1.5 mL of methanol into a measuring flask which was filled up with water to 3 mL.

2.4.2 DON

After addition of 10 g PEG 8000 (Fluka, Buchs, Switzerland) ground samples (25 g) were extracted with 200 mL of water and mixed for 3 min using a high-speed Ultra-Turrax homogenizer (Janke & Kunkel IKA-Labortechnik). The extract was filtered through a DONtest® fluted filter (Vicam, Watertown, MA, USA). Two milliliters of this extract was passed through an immunoaffinity column (DONPREP®, R-Biopharm). After washing the column with 20 mL of water, the toxins were eluted with 1.5 mL of methanol. The eluate was evaporated to dryness under a gentle nitrogen stream and redissolved in 1 mL of water/methanol 85 : 15 v/v.

2.4.3 OTA

Ground samples (25 g) were extracted with ACN/water (60 : 40 v/v) and treated for 2 min using a high-speed Ultra-Turrax homogenizer (Janke & Kunkel IKA-Labortechnik). The extract was cleaned by a Whatman No. 4 filter paper (Whatman) and 4 mL of this extract were diluted with 44 mL of PBS-buffer (pH 7.4, Oxoid, Hampshire, England) and passed through an immunoaffinity column (OCHRAPREP®, R-Biopharm). After washing the column with 20 mL of PBS-buffer the toxins were eluted with 1.5 mL of methanol/glacial acetic acid (Fluka) 98 : 2 v/v, into a measuring flask which was filled up with water to 3 mL.

2.4.4 FUM (B_1 and B_2)

Ground samples (50 g) were extracted with methanol/0.05 M hydrochloric acid (80 : 20 v/v) by shaking for 30 min. The extract was filtered through a fluted filter (Vicam) and 10 mL of the filtrate were diluted to 50 mL with PBS-buffer (pH 7.4, Oxoid) and filtered again through a Whatman GF/A glass microfiber filter paper (Whatman). Ten milliliters of this solution was cleaned up using an immunoaffinity column (FUMONITEST®, R-Biopharm). The column was washed with 10 mL of PBS-buffer and toxins were eluted with 1.5 mL of methanol. The eluate was evaporated to dryness under a gentle stream of nitrogen and redissolved in 0.5 mL of ACN/water 50 : 50 v/v.

2.5 Instrumental conditions

The HPLC apparatus consisted of a Merck/Hitachi (Darmstadt, Germany) L-7100 low-pressure gradient pump equipped with an L-7360 column-oven and an L-7200 autosampler. For detection an L-7455 DAD and an L-7480 fluorescence detector was used. To confirm results obtained for DON and OTA a postcolumn-derivatization unit (Pickering Laboratories, Mountain View, CA, USA), combined with the described HPLC apparatus, was applied.

2.5.1 ZEA

Analytical column: Synergi™ Hydro-RP 80 Å, 250 mm × 4.6 mm id, 4 µm (Phenomenex, Aschaffenburg, Germany); solvent with isocratic flow: methanol/water 75:25, v/v, 0.9 mL/min; oven temperature: 30°C; injection volume: 100 µL; fluorescence detection: λ_{ex} = 446 nm, λ_{em} = 274 nm.

2.5.2 DON

Analytical column: Aqua® C18 125 Å, 250 mm × 4.6 mm id, 5 µm (Phenomenex); solvent with isocratic flow: water/ACN 85 : 15 v/v, 1.0 mL/min; oven temperature: 30°C; injection volume: 100 µL; diode-array detection (UV): λ = 218 nm.

Parameters for postcolumn-derivatization: analytical column: Aqua® C18 125 Å, 250 mm × 4.6 mm id, 5 µm (Phenomenex); solvent with isocratic flow: 0.01 M aqueous acetic acid/ACN 90 : 10, v/v, 0.8 mL/min; oven temperature: 35°C; injection volume: 100 µL; fluorescence detection: λ_{ex} = 360 nm, λ_{em} = 470 nm; hydrolysis reagent: 0.15 M aqueous sodium hydroxide; derivatization reagent: 0.03 M aqueous methylacetoacetate (VWR, Darmstadt, Germany) and 2 M aqueous ammoniumacetate (VWR); reactor temperature: 115°C.

2.5.3 OTA

Analytical column: Lichrospher® 100 RP-18, 250 mm × 4 mm ID, 5 µm (Merck); solvent with isocratic flow: methanol/water 50 : 50 v/v, 1.0 mL/min; oven temperature: 25°C; injection volume: 100 µL; fluorescence detection: λ_{ex} = 330 nm, λ_{em} = 460 nm.

Parameters for postcolumn-derivatization: analytical column: Lichrospher® 100 RP-18, 250 mm × 4 mm id, 5 µm (Merck); solvent with isocratic flow: ACN/water/glacial acetic acid 49.5 : 49.5 : 1 v/v, 0.8 mL/min; oven temperature: 25°C; injection volume: 100 µL; fluorescence detection: λ_{ex} = 390 nm, λ_{em} = 440 nm; reagent: 12.5% aqueous ammonia; reactor temperature: 40°C.

2.5.4 FUM (B_1 and B_2)

Analytical column: Synergi Polar-RP 80 Å, 150 mm × 4.6 mm id, 4 µm (Phenomenex); binary gradient of a methanol/phosphate-buffer (pH 3.3) for solvent A with 65 : 35 v/v and for solvent B with 80 : 20 v/v; gradient: 0 min 100% solvent A; 3 min 100% solvent A; 4 min 50% solvent A; 6 min 50% solvent A; 7 min 40% solvent A; 8 min 40% solvent A; 11 min 30% solvent A; 15 min 20% solvent A; 17 min 100% solvent A; 26 min 100% solvent A; flow: 1.0 mL/min; oven temperature: 35°C; injection volume: 10 µL; fluorescence detection: λ_{ex} = 330 nm, λ_{em} = 450 nm.

Parameters for precolumn-derivatization (performed by special programming of the autosampler): reagent: aqueous solution of each 1% *ortho*-phthalaldehyde (Fluka) and 2-mercaptoethanol (Fluka), 17% methanol and 3.2% sodium tetraborate-decahydrate (Merck); injection volume of reagent: 10 µL; reaction time: 3 min.

3 Results and discussion

3.1 DON, ZEA, and OTA in cereals and cereal products

Maximum levels set for cereal grains and processed cereal products intended for direct human consumption are 500 µg/kg for DON (baby food 100 µg/kg), 50 µg/kg for ZEA (baby food 20 µg/kg) [3], and 3 µg/kg for OTA (baby food 0.5 µg/kg) [2].

From the 77 samples analyzed in 2004 (Table 1), only one rye sample was highly contaminated with 5072 µg/kg ZEA. Investigations showed that this sample was an unprocessed product and had been taken directly from the field during harvest and therefore was not intended for direct human consumption. Additionally it was stored improperly under warm conditions. About 27% of all other samples were contaminated with one or more mycotoxins; however, mycotoxin levels of all samples did not exceed the maximum levels. The highest values for DON were found in wheat and for OTA in rye. The contamination rate of baby food was very low (8%) and only one sample was contaminated with ZEA (4 µg/kg).

3.2 FUM in maize meal and semolina

In Germany a maximum level of 500 µg/kg for the sum of FUM B_1 and B_2 for maize meal and semolina and processed maize products intended for direct human consumption was set [3].

In contrast to most of the results obtained during the year 2003, in 2004 both a very high contamination rate of 92% (Table 1) and high levels of FUM were found for maize

meal and semolina (Fig. 1). In 46% of the samples the levels were below 100 µg/kg. The highest FUM levels were found in maize meal samples from Turkey with a total of 6617 µg/kg, the 13-fold of the maximum level of 500 µg/kg, and from Italy with a total of 2739 µg/kg, the five-fold concentration of this maximum level (Fig. 2). About 30% of the samples analyzed in 2004, which originate from the harvest of 2003, exceeded the maximum level.

Table 1. Results from 77 samples of cereal and cereal products on DON-, ZEA- and OTA-contamination and from 37 samples of maize meal and semolina on FUM-contamination in 2004

Foodstuff	Toxin	Analyzed samples	Positive samples	Cases of noncompliance	Mean value (µg/kg)	Maximum value (µg/kg)
Infant food (grain mash and rusk)	DON	13	0	0	<LOQ	<LOQ
	ZEA	13	1 (7.7%)	0	n.c.	4.0
	OTA	–	–	–	–	–
Oat flakes	DON	7	1 (14%)	0	n.c.	192
	ZEA	7	0	0	<LOQ	<LOQ
	OTA	7	0	0	<LOQ	<LOQ
Hulled wheat and barley	DON	2	0	0	<LOQ	<LOQ
	ZEA	2	0	0	<LOQ	<LOQ
	OTA	1	0	0	<LOQ	<LOQ
Buckwheat and unripe spelt grain	DON	3	0	0	<LOQ	<LOQ
	ZEA	2	0	0	<LOQ	<LOQ
	OTA	2	0	0	<LOQ	<LOQ
Rye	DON	13	0	0	<LOQ	<LOQ
	ZEA	6	1 (17%)	1 (17%)	n.c.	5072
	OTA	10	6 (60%)	0	0.5	1.7
Wheat	DON	16	9 (63%)	0	138	445
	ZEA	15	1 (6.7%)	0	n.c.	23
	OTA	–	–	–	–	–
Barley	DON	1	1 (100%)	0	n.c.	56
	ZEA	1	0	0	<LOQ	<LOQ
	OTA	1	0	0	<LOQ	<LOQ
Other processed cereal products	DON	23	3 (13%)	0	22	56
	ZEA	23	7 (30%)	0	1.0	<LOQ
	OTA	23	3 (13%)	0	<LOQ	<LOQ
Maize meal and semolina	FUM	37	34 (92%)	11 (30%)	823	6617

LOQ, DON 50 µg/kg; ZEA 4 µg/kg, OTA 0.1 µg/kg, and FUM 5 µg/kg.
–, Not analyzed.
n.c., Not calculated, only one positive sample.

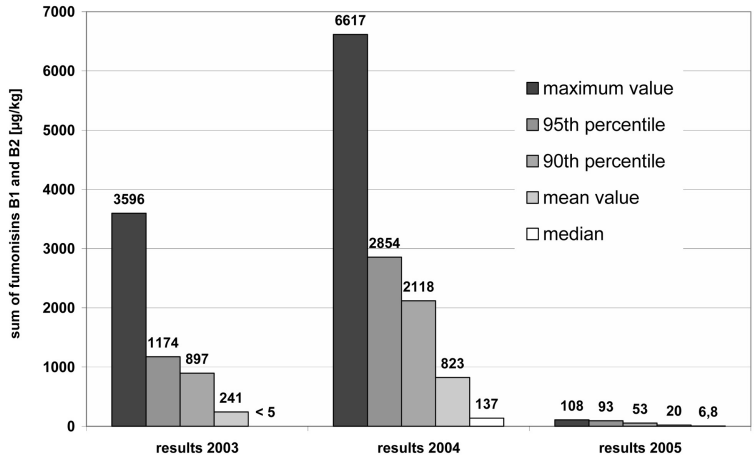

Figure 1. Comparison of contamination rates of maize meal and semolina analyzed from 2003 to 2005.

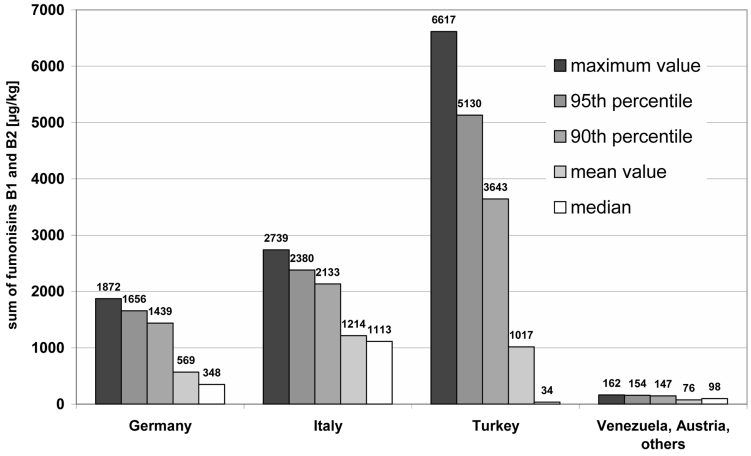

Figure 2. Contamination with FUM of maize meals and semolina from different countries in 2004 (the origin does not inevitably designate the geographical origin of the maize grains, but only the country of origin of the manufacturer or packager).

4 Concluding remarks

None of the cereals and cereal products intended for direct human consumption which were analyzed in 2004 for ZEA, DON, and OTA, exceeded the maximum levels, and therefore these samples did not represent a health risk for the consumer.

In contrast, FUM levels of maize meals and semolina analyzed in 2004 were permanently very high. Comparison of the results obtained for 2004 with that obtained in 2003 showed that most FUM-contents measured in 2003 were significantly lower with maximum levels up to 1450 µg/kg (Fig. 1). Since in autumn 2003 two maize meal samples with unknown origin had already very high FUM-contents with more than 3500 µg/kg, it is assumed that the reason for the high FUM levels of maize meal from the regions concerned measured in 2004 is based on the extremely warm weather in 2003, which is known to facilitate FUM-production [5]. This assumption is also supported by the results obtained till now for 2005, which show a low FUM-contamination of maize meal and semolina with FUM levels not exceeding 100 µg/kg (Fig. 2). Very high FUM-contaminations rates and contents were also described by other authors in 2004 (harvest 2003) for maize meal, semolina, and tortilla chips with maximum values up to 11 700 µg/kg [6].

Concerning the toxicological evaluation of the highest contamination level with 6617 µg/kg FUM no critical dietary intakes were assumed for the entire population with mean consumption habits in Germany. For specific groups, however, *e.g.*, for consumers preferring maize meal because of dietetic reasons (intolerance to the protein gluten and celiac disease) dietary intakes exceeding the TDI of 2 µg/kg body weight established by the SCF (Scientific Committee on Food, http://www.europa.eu.int/comm/food/fs/sc/scf/out123_en.pdf) could be supposed. Therefore, the highest value was evaluated as harmful to health (article 14, para. 2a, Commission Regulation (EC) No. 178/2002 [7]), especially for children.

5 References

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