Occurrence of ochratoxin A and citrinin in Czech cereals and comparison of two HPLC methods for ochratoxin A detection

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The aims of the study were to obtain information about the occurrence of ochratoxin A (OTA) and citrinin (CIT) in cereals harvested in the Czech Republic and to compare two analytical procedures for detecting OTA. A total of 34 cereal samples, including two matrix reference materials (R-Biopharm, Germany), were analysed. The results were compared with the limit for raw cereal grains used as a foodstuff according to Commission Regulation No. 1881/2006, which allows a maximum OTA level of 5 $\mu g\,kg^{-1}$. Compared were two methods based on the high-performance liquid chromatography principle, one using the immunoaffinity columns OchraTest™ (VICAM) and the second based on solvent partition (PART), both followed by fluorescence detection. The highest OTA contents were found in two barley samples. According to the method employed, the results for the first sample (malting barley) were VICAM = 31.43 $\mu g\,kg^{-1}$ and PART = 44.74 $\mu g\,kg^{-1}$. For the second sample (feeding barley) they were VICAM = 48.63 $\mu g\,kg^{-1}$ and PART = 34.40 $\mu g\,kg^{-1}$. Two samples of bread wheat had an OTA content approaching the legal limit (VICAM = 4.71 $\mu g\,kg^{-1}$ and PART = 6.03 $\mu g\,kg^{-1}$; VICAM = 4.12 $\mu g\,kg^{-1}$ and PART = 3.95 $\mu g\,kg^{-1}$). CIT was analysed using the PART method only, and its highest content (93.64 $\mu g\,kg^{-1}$) was found for the malting barley sample with high OTA content (44.74 $\mu g\,kg^{-1}$ as analysed using PART).

Keywords: high-performance liquid chromatography (HPLC); mycotoxins; ochratoxin A; cereals

Introduction

On a world scale, five mycotoxins or groups of mycotoxins are considered to be important in human health. These are aflatoxins, ochratoxin A (OTA), fumonisins, trichothecenes, and zearalenone (Miller 1996). OTA is the only toxin among them produced by *Penicillium* species. It can also be produced by several species of the genus *Aspergillus*: *A. ochraceus* (now named *Aspergillus alutaceus*), *A. carbonarius* and *A. niger* (Pitt 1987; Madhyastha et al. 1990). *P. verrucosum* has been identified as the only producer of OTA in cereals in temperate climates (Ciegler et al. 1973; Lund and Frisvad 2003; Scudamore 2005). Another mycotoxin, citrinin (CIT), can occur in cereals together with OTA, as *P. verrucosum* is able to produce both of these mycotoxins, but CIT production is also possible by other *Penicillium* (Scott et al. 1972; Pitt 1987; Abramson et al. 1990) and *Aspergillus* species (Scott 1977; Bettina 1984). Simultaneous contamination with OTA and CIT of both raw cereals (Abramson et al. 1999; Vrabcheva et al. 2000) and cereal-based products (Molinié et al. 2005) has been identified.

The International Agency for Research on Cancer (IARC) (1993) classifies OTA as possibly carcinogenic to humans (Group 2B) based on sufficient evidence of carcinogenicity in experimental animals. OTA has nephrotoxic effects, and it has been shown to cause mycotoxic porcine nephropathy (Krogh 1976). It has other adverse effects that include mutagenicity (DeGroene et al. 1996; Palma et al. 2007) and genotoxicity (Creppy et al. 1985; Pfohl-Leszkowicz et al. 1991; Lebrun and Föllmann 2002; Pfohl-Leszkowicz and Manderville 2007). CIT is also nephrotoxic (Krogh et al. 1973; Frank 1992) and genotoxic (Segvic-Klaric et al. 2007; Iwahashi et al. 2007; Pfohl-Leszkowicz et al. 2008). It enhances OTA renal toxicity in pigs (Krogh et al. 1973), the incidence of renal cell tumours in male mice (Kanizawa 1984), and kidney adenomas in male rats (Arrai and Hibino 1983), although CIT alone has not been

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proven carcinogenic. Both OTA and CIT seem to be implicated in Balkan endemic nephropathy in some Balkan areas (Petkova-Bocharova and Castegnaro 1991; Vrabcheva et al. 2000; Pfohl-Leszkowicz et al. 2002, 2007). The incidence of and mortality from urothelial urinary tract tumours have been correlated with the geographical distribution of Balkan endemic nephropathy in Bulgaria and Yugoslavia. A relatively high frequency of contamination of cereals and bread with ochratoxin A has been reported in an area of the former Yugoslavia where Balkan endemic nephropathy is present (IARC 1976, 1983, 1987, 1993). OTA–DNA adducts were found in kidney, liver, and spleen of mice and rat orally exposed to OTA (Pfohl-Leszkowicz et al. 1991, 1993) and also in pig fed OTA contaminated food (Miljkovic et al. 2002).

Due to documented negative effects on human health, OTA content is limited in unprocessed cereals intended for food production (the limit is $5\,\mu g\,kg^{-1}$) and in products derived from raw cereals, including processed cereal products and cereals intended for direct human consumption (the limit is $3\,\mu g\,kg^{-1}$) and also in baby food (the limit of $0.5\,\mu g\,kg^{-1}$) (European Commission 2006a). In cereals and cereal products intended for animal feeding, only guidance values are given: for cereals and cereal products it is $0.25\,mg\,kg^{-1}$, but there are different values for complementary and complete feedstuffs for pigs ($0.05\,mg\ kg^{-1}$) and for poultry ($0.10\,mg\,kg^{-1}$) (European Commission 2006b). No limits have been established for CIT.

OTA is fairly stable to heat. In cereal products, up to 35% of the toxin survives autoclaving for up to 3 h (IARC 1976). It currently can be found in cereal-based products (Wolff 2000; Lombaert et al. 2003) also together with CIT (Molinié et al. 2005). Beer, through malting barley or other kinds of cereals used for malt production, could also be contaminated with OTA (Tangni et al. 2002; Anselme et al. 2006). As Harcz et al. (2007) conclude, there is a risk of exposure to a significant amount of OTA with respect to the tolerable daily intake (TDI) especially in such countries with high beer consumption habits as Belgium, the Czech Republic, the UK, Germany, Ireland, Austria, and Denmark.

Based on risk assessment performed by the Joint FAO/WHO Expert Committee on Food Additives (JECFA), cereals and cereal products contribute more than 50% of human OTA exposure (JECFA 2001). Lund and Frisvad (2003) report that cereals normally account for 50–80% of the average consumer intake of OTA. A basic and reasonable way to reduce the consumer intake of OTA is to monitor it thoroughly in raw cereals and to prevent its entry into the food chain.

Frisvad and Viuf (1986) investigated 70 samples of barley from Denmark and found OTA content varying from zero to $7380\,\mu g\,kg^{-1}$. According to reports of Scott et al. (1972) and Krogh et al. (1973), detected OTA contamination levels in cereals range from 0.03 to 27.5 ppm. As Scott (1994) reported, concentrations of CIT are often several times higher than are those for accompanying OTA. A detailed survey of OTA incidence in cereals in Europe by Rizzo et al. (2002) found that the highest reported levels were in wheat (239 samples analysed) and rye (228 samples analysed) from Poland, and a maximum level for both cereals was $2400\,\mu g\,kg^{-1}$. Birzele et al. (2000) observed the occurrence of OTA using the enzyme-linked immunoabsorbant assay (ELISA) method within the limit of detection (LOD) of $0.4\,\mu g\,kg^{-1}$ for 43 freshly harvested wheat samples in Germany. They found 21% positive samples and a maximum estimated value of $0.7\,\mu g\,kg^{-1}$. Conkova et al. (2006) examined 105 cereal samples which were taken immediately after harvest from selected localities of Poland (45 samples) and eastern Slovakia (60 samples). OTA was analysed using the high-performance liquid chromatography (HPLC) method with the LOD of $0.015\,\mu g\,kg^{-1}$ and no positive sample was found. In Belgium, cereal grain samples were reported to be contaminated up to $25.4\,\mu g\,kg^{-1}$ (Chandelier et al. 2004). Malir et al. (2006) studied the occurrence of OTA in 114 cereal samples taken at the time of harvest in the Czech Republic, among which ten were positive (the LOD of the method used was $0.4\,\mu g\,kg^{-1}$) and the maximum level was $37.0\,\mu g\,kg^{-1}$. Analysis of 125 wheat samples from France revealed the presence of OTA in 60% of samples ranging from trace to $69\,\mu g\,kg^{-1}$. The highest amount was found in a sample collected in a farm located in northern part of France and badly stored (Bédouret et al. 2001; Pfohl-Leszkowicz et al. 2007). Half of the samples contaminated by OTA were also contaminated by CIT.

The reliability and comparability of analytical results for monitoring studies are crucially important. The percentage of reported positive samples depends on the LOD of the method employed. Several methods for OTA analyses in raw cereals have been developed and validated (Visconti and DeGirolamo 2005). The methods are mainly based on thin-layer chromatography (Nesheim et al. 1973; Levi 1975) or, now more frequently, on liquid chromatography (LC) with fluorescence detection without (Nesheim et al. 1992) or with immunoaffinity clean-up (IMA) (Entwisle et al. 2000). For simultaneous analyses of OTA and CIT, a method suitable for wheat and barley was described by Lepom (1986), and a versatile method suitable for raw cereals and cereal products using partition was developed by Molinié et al. (2005). In the method of Molinié et al., the mycotoxins are extracted by acetonitrile instead of chloroform and at a lower pH (1.5 versus 4.0). These modifications are particularly important for CIT for which the recovery reached 80% in the method of Molinié et al. and is only 60% with chloroform. Purification on immunoaffinity could lead

to underestimation, notably when OTA has been extracted in alkaline conditions, because it is converted onto open-ring OTA which could not cross-react with antibodies anymore (Pfohl-Leszkowicz et al. 2006; Castegnaro et al. 2006). The current status of methods for OTA detection and of methods development is reviewed in detail by Shephard et al. (2009).

Methods based on antigen–antibody reaction, including ELISA, have also been developed for both OTA and CIT analysis. Numerous procedures have been published that use monoclonal or polyclonal antibodies in raw cereals, e.g. for OTA in barley (Morgan et al. 1983) or in wheat (Lee and Chu 1984). Currently, an entire range of ELISA microtitre plate kits is sold which is intended for both OTA and CIT detection in cereals, feeds, beer and other matrices.

The aims of the current study were to detect the levels of contamination with OTA and CIT in unprocessed cereals harvested in the Czech Republic and to compare analytical procedures based on LC for OTA analysis in raw cereal grain with and without immunoaffinity clean-up.

Materials and methods

Sample collection

Set I

The first set of samples (numbers 1–10) includes ten samples of freshly harvested winter wheat (2006 harvest) selected from samples provided by farmers to a laboratory of Agrotest fyto, s.r.o. for analysis in relation to monitoring the quality of wheat intended for bread-making harvested in the Czech Republic. Samples delivered at the end of monitoring (i.e. later than 25 August 2006) were selected. The samples were collected by farmers who had been instructed on the principles of good sampling according to Commission Regulation (EC) No. 401/2006 specifying the methods of sampling and analysis for the official control of mycotoxins levels in foodstuffs (European Commission 2006c). After delivering to the laboratory, wheat moisture content was determined according to Commission Regulation (EC) No. 824/2000 of 19 April 2000 establishing procedures for the taking-over of cereals by intervention agencies and laying down methods of analysis for determining the quality of cereals (European Commission 2000). The samples were then left in their original packing and stored in a dry environment at 15–18°C until OTA analyses in November–December 2006. Nine of the ten samples analysed were samples of bread wheat and one sample was wheat for feeding. The characteristics of the samples (harvest date, delivery date, and moisture content) together with results are given in Table 1.

Set II

The second set (numbers 11–34) contains 22 raw cereal grain (twelve wheat and ten barley) samples, all but two (numbers 11 and 12) of which were taken from storage facilities, and two matrix reference materials. Sample 11 was naked barley intended for producing wholemeal foods originating from organic farming. It was taken from a tractor trailer on a farm where it had been temporarily stored. Barley sample 12 was taken from a lot offered to a malt house for malt production. Having checked its malting quality in the malt house laboratory, it had been refused due to a higher content of admixtures and impurities, as well as a mouldy smell. The other 20 samples were collected from randomly selected storage facilities across the Kromeriz district and sampled by warehousers according to Commission Regulation (EC) No. 401/2006. Sampling and analyses were carried out during November–December 2008. The samples are characterised and the results presented in Table 2. Reference materials (RM) (wheat meal P64/OW803 with the determined OTA value of $1.8 \pm 0.6\,\mu g\,kg^{-1}$ and P64/OW806 with the determined OTA value of $7.7 \pm 2.0\,\mu g\,kg^{-1}$) were purchased from R-Biopharm AG (Darmstadt, Germany).

Methods of analyses, chemicals

HPLC-VICAM method (VICAM)

Analyses were performed by a laboratory of the State Veterinary Institute in Olomouc (Czech Republic), which is accredited for this determination according to EN ISO/IEC 17025:2005 being employed for official control of OTA levels. OchraTest™ immunoaffinity columns were used. Guidelines from the manufacturer's of VICAM – OchraTest™ (Watertown, MA, USA) and the OchraTest™ WB HPLC Instruction Manual (VICAM 2005), paragraph 4.3 HPLC Procedure for Corn, Milo & Feeds, were followed. Also used were a methanol : water (80 : 20) extraction mixture, 4.6×75 mm Symmetry C18 column (Waters, Milford, MA, USA), C18 sorbent, flow rate of 0.8 ml min^{-1} at 25°C. An Agilent 1100 fluorescence detector was used (Santa Clara, CA, USA), with excitation and emission wavelengths of 333 and 477 nm, respectively. Chemicals were of HPLC grade and purchased from Sigma Aldrich (Czech Republic). The method validation in the laboratory was carried out at the extent needed to comply with EN ISO/IEC 17025:2005 requirements. Performance characteristics are summarised in Table 3. Recovery was determined using a repeated analysis of three spiked wheat samples at the OTA concentrations of 2.5, 5.0 and $10.0\,\mu g\,kg^{-1}$, which was carried out on one day by the same operator. The average recovery for these three concentrations was $87.0\% \pm 6.1\%$. All results are corrected for this recovery. A limit of

Table 1. Ochratoxin A and citrinin in cereals harvested in the Czech Republic, Set I, samples from farmers, 2006 harvest, analyses in November–December 2006 (VICAM – method using the immunoaffinity columns OchraTest™; PART – method based on solvent partition without purification on immunoaffinity columns).

Number	Sample description	Date of:		Moisture content[a] (%)	Ochratoxin A ($\mu g\,kg^{-1}$)		Citrinin ($\mu g\,kg^{-1}$) PART	Percentages of admixtures and impurities[b]			
		Harvest	Delivery to the laboratory		VICAM	PART		Category 1	Category 2	Category 4	Category 5
1	Bread wheat	22 August	25 August	11.31	<LOD	<LOD	<LOD	0.88	1.58	32.74	0.46
2	Bread wheat	3 August	29 August	12.05	4.71 ± 1.18	6.03 ± 1.51	<LOQ	1.45	1.37	0.00	4.82
3	Bread wheat	26 August	30 August	12.21	<LOD	<LOD	<LOD	1.68	4.54	9.73	0.26
4	Wheat for feeding	24 August	30 August	12.03	<LOD	<LOQ	<LOD	4.48	2.17	47.60	2.51
5	Bread wheat	24 August	6 September	11.82	<LOD	<LOD	<LOD	3.68	0.32	25.36	2.02
6	Bread wheat	5 September	18 September	11.74	<LOD	<LOD	<LOD	7.69	0.95	15.60	2.18
7	Bread wheat	1 September	19 September	12.41	<LOD	<LOD	<LOD	0.89	5.20	0.65	2.01
8	Bread wheat	1 September	5 October	11.25	<LOD	<LOD	<LOD	0.43	2.96	1.09	1.75
9	Bread wheat	27 August	6 October	10.90	<LOD	<LOD	<LOD	1.63	2.07	0.78	1.69
10	Bread wheat	6 September	13 October	11.38	<LOD	<LOQ	<LOD	4.78	0.42	10.90	1.67

Notes: [a]Moisture content at the time of a sample's delivery to the laboratory.
[b]Categories of materials that are not basic cereal of unimpaired quality, according to Commission Regulation (EC) No. 824/2000: 1, broken grains; 2, impurities consisting of grains; 4, sprouted grains; and 5, miscellaneous impurities.

Table 2. Ochratoxin A and citrinin in cereals harvested in the Czech Republic, Set II, samples from storage facilities, analyses in November–December 2008 (VICAM – method using the immunoaffinity columns OchraTest™; PART – method based on solvent partition without purification on immunoaffinity columns).

Number	Sample description	Harvest year	Ochratoxin A ($\mu g\,kg^{-1}$)		Citrinin ($\mu g\,kg^{-1}$)	Percentage of admixtures and impurities[e]			
			VICAM	PART	PART	Category 1	Category 2	Category 4	Category 5
11	Naked barley, foodstuff[a]	2008	0.49 ± 0.12	0.46 ± 0.12	< LOQ	1.37	1.16	0.00	0.00
12	Malting barley[b]	2007	31.43 ± 7.86	44.74 ± 11.19	93.64 ± 28.09	1.53	1.04	0.00	14.35
13	Barley for feeding	2007	0.67 ± 0.17	0.35 ± 0.09	< LOQ	0.90	0.69	0.00	0.22
14	Barley for feeding	2007	< LOD	< LOD	< LOD	2.58	4.11	0.00	0.53
15	Barley for feeding	2007	< LOD	0.58 ± 0.15	< LOD	27.52	15.38	0.00	6.09
16	Barley for feeding	2007	2.52 ± 0.63	1.03 ± 0.26	5.25 ± 1.58	3.47	6.41	0.00	1.06
17	Barley for feeding	2007	< LOD	< LOD	< LOD	0.85	0.71	0.00	0.11
18	Malting barley	2007	< LOD	< LOD	1.82 ± 0.46	2.34	2.25	0.00	0.20
19	Malting barley	2007	< LOQ	< LOQ	< LOQ	4.09	1.93	0.00	0.15
20	Barley for feeding	2007	48.63 ± 12.16	34.40 ± 8.60	13.17 ± 3.29	18.21	3.35	0.00	3.91
21	Bread wheat	2005	4.12 ± 1.03	3.95 ± 0.99	< LOQ	2.20	4.00	0.00	5.34
22	Wheat for feeding	2007	< LOQ	< LOQ	< LOD	6.37	3.09	0.00	0.85
23	Wheat for feeding	2007	< LOD	0.26 ± 0.07	< LOD	2.64	3.84	0.00	0.61
24	Wheat for feeding	2007	< LOD	< LOD	< LOD	5.42	5.39	0.00	1.17
25	Bread wheat	2007	0.33 ± 0.08	0.30 ± 0.08	< LOD	3.98	1.64	0.00	1.92
26	Wheat for feeding	2007	< LOD	< LOQ	< LOD	5.49	0.39	0.00	1.61
27	Wheat for feeding	2007	< LOD	< LOD	< LOD	1.92	2.09	0.05	0.80
28	Wheat for feeding	2007	< LOD	< LOD	< LOD	2.39	1.54	0.00	0.77
29	Wheat for feeding	2007	< LOD	< LOQ	< LOD	1.07	1.71	0.00	0.26
30	Wheat for feeding	2007	< LOD	< LOQ	< LOD	4.97	4.06	0.06	0.95
31	Wheat for feeding	2007	< LOD	< LOD	< LOD	15.68	5.71	0.00	0.68
32	Wheat for feeding	2007	< LOD	< LOQ	< LOD	17.49	4.09	0.00	0.53
33	Reference material[c]	–	8.75 ± 2.19	12.73 ± 3.18	< LOD	–	–	–	–
34	Reference material[d]	–	2.54 ± 0.64	4.52 ± 1.13	< LOD	–	–	–	–

Notes: [a]Sample obtained from organic farming.
[b]Sample obtained from a malt house laboratory.
[c]P64/OW806 (7.7 ± 2.0)$\mu g\,kg^{-1}$, wheat.
[d]P64/OW803 (1.8 ± 0.6)$\mu g\,kg^{-1}$, wheat.
[e]Categories of materials that are not basic cereal of unimpaired quality, according to Commission Regulation (EC) No. 824/2000: 1, broken grains; 2, impurities consisting of grains; 4, sprouted grains; and 5, miscellaneous impurities.

Table 3. Performance characteristics of two HPLC methods used for analyses of ochratoxin A (OTA) (VICAM – method using the immunoaffinity columns OchraTest™; PART – method based on solvent partition without purification on immunoaffinity columns).

Method	Recovery (%)	Repeatability (%)	Inter-day variability (%)	Limit of detection (LOD) ($\mu g\,kg^{-1}$)	Limit of quantification (LOQ) ($\mu g\,kg^{-1}$)
VICAM	87.0 ± 6.1	7.2	12.0	0.15	0.25
PART	75.6 ± 6.6	7.5	12.5	0.05	0.20

quantification (LOQ) of $0.25\,\mu g\,kg^{-1}$ and a limit of detection (LOD) of $0.15\,\mu g\,kg^{-1}$ were calculated based on a blank sample signal when a value of maximum variation in a chromatogram basic line for the blank sample in the region given by a 20-multiple of OTA peak half-width and calibration line slope were used for calculation. The average repeatability of 7.2% was determined from five repeated measurements of spiked samples at the concentrations of 2.5, 5.0 and $10.0\,\mu g\,kg^{-1}$ performed by the same operator on one day and with the same HPLC system. Inter-day variability was measured on the same set of samples on five successive days resulting in a reproducibility of 12.0%. All samples were measured in duplicate and the results are presented as mean ± measurement uncertainty, which was estimated at 25%. For the purposes of this contribution, results obtained using this method are considered to be reference results, meaning that if only one final OTA level is shown in the text, and it is not specified in any other way, then it is from HPLC-VICAM analysis.

HPLC method based on solvent partition (PART)

Analyses were performed by a Laboratory of Chemical Engineering UMR CNRS/INPT/UPS n° 550 in Toulouse (France). The method according to Molinié et al. (2005) was employed. All reagents were of HPLC grade and purchased from ICS (France). Deionized water was used to prepare all aqueous solutions and for HPLC. OTA free of benzene and CIT were from Sigma Chemicals (France). The HPLC analysis used a Gilson 811B dynamic chromatography pump coupled to a Spectra Physics 2000 fluorescence spectrophotometer and an ICS autosampler. In order to optimise sensitivity for the analysis of OTA and CIT, which have different excitation and emission fluorescence parameters (OTA of 335 and 465 nm, CIT of 331 and 500 nm, respectively), analyses were performed using HPLC conditions adapted for each toxin. A C18 spherisorb column (3 μm C18, 46 × 250 mm) was used, preceded by a C18 pre-column from ICS. To reduce the risk of false-positives, the system was run isocratically using two different phases for the analyses of OTA. Phase 1 was methanol/acetonitrile/sodium acetate (5 mM)/acetic acid (300/300/400/28); flow rate was $0.7\,ml\,min^{-1}$; and the elution time of OTA was about 9 min. Phase 2 was H_3PO_4 (0.33 M)/acetonitrile/propan-2-ol (600/400/50); flow rate was 0.7 min, and the elution time of OTA was about 18 min. A third phase was used for determining CIT: H_3PO_4 (0.33 M)/acetonitrile/propan2-ol (700/300/50); flow rate was 0.7 min; and the elution time of CIT was about 19 min. Validation of this method was carried out and validation data have been published previously (Molinié et al. 2005). Performance characteristics are summarised in Table 3 in comparison with the VICAM method. OTA recovery was calculated based on analysis of five different cereal samples spiked with $3\,\mu g\,kg^{-1}$ (corresponding to value from European Union legislation) and $10\,\mu g\,kg^{-1}$ on the same day, by the same operator and with the same HPLC system. The average OTA recoveries were $76.1\% \pm 5.7\%$ and $75.1\% \pm 11.9\%$, respectively, for cereal samples spiked with OTA at the concentration levels of 3 and $10\,\mu g\,kg^{-1}$. The results presented are corrected for an average recovery $75.6\% \pm 6.6\%$. OTA repeatability was calculated based on the results of these samples analysed five times on the same day and amounts to 7.5%. OTA inter-day variability was measured by the means of samples spiked with $3\,\mu g\,kg^{-1}$ and one naturally contaminated sample analysed on five successive days by the same operator and with the same HPLC system resulting in a value of 12.5%. All samples were measured in duplicate and results are presented as mean ± measurement uncertainty, which was estimated to be 25%. The dose–response curve for OTA analysis was measured by analysis of solutions containing OTA in concentrations from $0.5\,\mu g\,l^{-1}$ to $1\,mg\,l^{-1}$ (corresponding to concentrations from $12.5\,ng\,kg^{-1}$ to $24.9\,\mu g\,kg^{-1}$ in cereals). The coefficient of linearity (R^2) was 0.997. The LOD was $0.05\,\mu g\,kg^{-1}$ and the LOQ was $0.20\,\mu g\,kg^{-1}$ at nine-times background. CIT recovery was calculated based on the analysis of five different cereal samples spiked with 3 and $10\,\mu g\,kg^{-1}$ on the same day by the same operator and with the same HPLC system. The average recovery was $80.3\% \pm 5.0\%$. The results are corrected for this recovery. CIT repeatability was calculated based on the analysis of these samples analysed five times on the same day on the level of 8.1%. For CIT inter-day variability calculation, samples spiked with $3\,\mu g\,kg^{-1}$ and one naturally contaminated sample were analysed on five successive days by the same operator and with

the same HPLC system. The average CIT reproducibility was 13.6%; the measurement uncertainty was estimated to be 30%. The dose–response curve for CIT was measured by analysis of solutions containing CIT in concentrations from 50 $\mu g\,l^{-1}$ to 2.25 $mg\,l^{-1}$ (corresponding to concentrations from 1.12 to 56.25 $\mu g\,kg^{-1}$ in cereals). R^2 was 0.98. The LOD was 0.5 $\mu g\,kg^{-1}$ and the LOQ was 1.5 $\mu g\,kg^{-1}$ at seven-times background. Recovery tests for both toxins simultaneously were carried out by means of analysing five different samples spiked with both toxins at the concentration of 3 $\mu g\,kg^{-1}$ each on the same day by the same operator with the same HPLC system. The average recovery was 81.3% ± 4.2% for OTA and 80.1% ± 5% for CIT. The confirmation of the presence of OTA in samples detected at 2 $\mu g\,kg^{-1}$ was performed using two techniques: the carboxypeptidase technique for producing α-ochratoxin (α-OT). Briefly, an aliquot taken from the purified extract of a sample where OTA was detected using the two HPLC methods was dried, and residue dissolved in 0.975 ml of a buffer solution of 0.04 M Tris-HCl, 1 M NaCl, pH 7.5. The amount of 25 µl of carboxypeptidase in water (100 U ml^{-1}) was added and the solution incubated at room temperature overnight. The samples were analysed by the HPLC chromatographic conditions used above for the analysis of OTA. The peak of OTA disappeared, whereas the peak of α-OT appeared. Some samples were analysed by LC-MS/MS by an external laboratory (data not shown).

Measurement of admixtures and impurities, and determination of moisture content

For all samples the parameter 'Matter other than basic cereals of unimpaired quality' was determined and for sample Set I also 'Moisture content'. Both parameters were analysed according to Commission Regulation (EC) No. 824/2000 of 19 April 2000 establishing procedures for the taking-over of cereals by intervention agencies and laying down methods of analysis for determining the quality of cereals (European Commission 2000). Values of 'Matter other than basic cereals of unimpaired quality' which were found for the samples were compared with maximum permitted values according to Commission Regulation (EC) No. 824/2000. For wheat they are as follows: 1, broken grains 5%; 2, impurities consisting of grains 7%; 4, sprouted grains 4%; and 5, miscellaneous impurities 3%. For barley, they are: 1, broken grains 5%; 2, impurities consisting of grains 12%; 4, sprouted grains 6%; and 5, miscellaneous impurities 3%.

Statistical analysis

Data were analysed by the Statistica program (Statsoft, Inc.), version 8.0. Measured values were plotted in a scatter plot with identity ($x = y$) and regression line; inter-method differences were correlated to measured VICAM values. A pairwise t-test and correlation analysis were used to determine the statistical significance of differences between the two methods. Values below the LOD were eliminated from a comparison. $p < 0.05$ was accepted as being significant (if not otherwise stated). Data are presented as mean ± measurement uncertainty (95% confidence interval).

Results

Sample analyses – Set I

The results are summarised in Table 1.

OTA analysis – VICAM method

Nine of the wheat samples analysed had an OTA content lower than the LOD of the method used (0.15 $\mu g\,kg^{-1}$). For sample 2, an OTA content of 4.71 ± 1.18 $\mu g\,kg^{-1}$ was found.

OTA and CIT analysis – PART method

Three samples were found to have an OTA content above the LOD of the method used (0.05 $\mu g\,kg^{-1}$). The highest OTA content was determined in sample 2 (6.03 ± 1.51 $\mu g\,kg^{-1}$), and two additional positive samples (numbers 4 and 10) had an OTA content between the LOD and the LOQ. CIT content between the LOD and the LOQ was found for sample 2; for all other samples CIT values were under the LOD.

Sample analyses – Set II

The results are summarised in Table 2.

OTA analysis – VICAM method

Using this method, nine samples were found to be OTA positive, of which two samples had OTA values between the LOD and the LOQ. The maximum level was for sample 20 (barley for feeding), at 48.63 ± 12.16 $\mu g\,kg^{-1}$. The second highest sample was number 12 (malting barley with poor quality parameters), with an OTA level of 31.43 ± 7.86 $\mu g\,kg^{-1}$. The third highest sample was bread wheat, number 21. Its OTA level was 4.12 ± 1.03 $\mu g\,kg^{-1}$. In analysing RM P64/OW806 with a determined value of 7.7 ± 2.0 $\mu g\,kg^{-1}$, the concentration 8.75 ±2.19 $\mu g\,kg^{-1}$ was found. In analysing RM P64/OW803 with a determined value of 1.8 ± 0.6 $\mu g\,kg^{-1}$, the concentration 2.54 ± 0.64 $\mu g\,kg^{-1}$ was found.

OTA and CIT analysis – PART method

Using this method, 15 samples were found to be OTA positive, of which six samples had OTA values between

the LOD and the LOQ. The maximum level was for sample 12 (malting barley with poor quality parameters), at $44.74 \pm 11.19\,\mu g\,kg^{-1}$. The second highest sample was number 20, with an OTA level of $34.40 \pm 8.60\,\mu g\,kg^{-1}$. The third highest sample was bread wheat, number 21, with an OTA level of $3.95 \pm 0.99\,\mu g\,kg^{-1}$. In analysing RM P64/OW806 with a determined value of $7.7 \pm 2.0\,\mu g\,kg^{-1}$, the concentration $12.73 \pm 3.18\,\mu g\,kg^{-1}$ was found. In analysing RM P64/OW803 with a determined value of $1.8 \pm 0.6\,\mu g\,kg^{-1}$, the concentration $4.52 \pm 1.13\,\mu g\,kg^{-1}$ was found. CIT content above the LOD ($0.5\,\mu g\,kg^{-1}$) was found for eight samples, of which four had CIT values between the LOD and the LOQ. The maximum CIT concentration ($93.6 \pm 28.09\,\mu g\,kg^{-1}$) was found for sample 12, which also had the highest OTA content as analysed using the same method.

Comparison of results obtained by VICAM and PART methods

For the 24 sample measurements (Set II samples and two RMs), *t*-test ($p = 0.20$) and correlation analysis showed no significant bias between the VICAM and the PART methods. Good correlation was observed between measured values ($r = 0.913$) (Figure 1), but not between inter-method differences and measured values ($r = 0.268$).

Discussion

Comparison of analytical procedures based on LC for OTA analysis in raw cereal grain with (VICAM) and without (PART) immunoaffinity clean-up

Two methods based on the HPLC principle, one using the immunoaffinity columns OchraTest™ (VICAM 2005) and the other based on solvent partition (Molinié et al. 2005), both followed by fluorescence detection, were used to analyse 32 samples of raw cereals and two

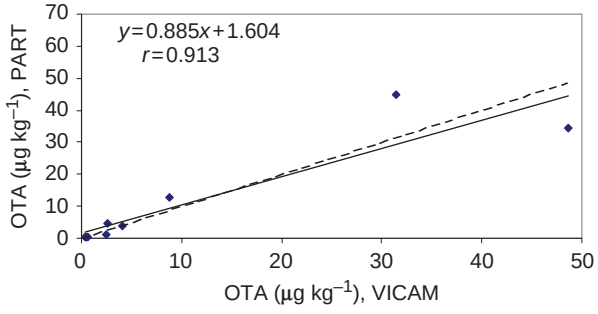

Figure 1. Scatter plot of OTA content measured by VICAM and PART method (VICAM – HPLC using the immunoaffinity columns OchraTest™; PART – based on solvent partition without purification on immunoaffinity columns). Dashed line = identity line.

reference materials (RMs). RMs P64/OW803 (OTA = $1.8 \pm 0.6\,\mu g\,kg^{-1}$) and P64/OW806 (OTA = $7.7 \pm 2.0\,\mu g\,kg^{-1}$) from R-Biopharm AG (Germany) were analysed. As the RMs used were not certified, only their properties such as homogeneity and stability could be taken into consideration and the results of both methods were compared only one with the other. The matrix of both RMs was wheat wholemeal. The values obtained using the VICAM method, 2.54 ± 0.64 and $8.75 \pm 2.19\,\mu g\,kg^{-1}$, respectively, were lower that the results of the PART method, $4.52 \pm 1.13\,\mu g\,kg^{-1}$ and $12.73 \pm 3.18\,\mu g\,kg^{-1}$ (Table 2). The reason could be that the recovery of OTA in acidic conditions is of 80%, whereas in neutral and alkaline conditions OTA is converted into open-ring OTA which is no more recognised by antibodies and thus are lost (Pfohl-Leszkowicz et al. 2006; Castegnaro et al. 2006). In the first set of samples, the VICAM method detected only one OTA positive sample, number 2, and the OTA level was determined to be $4.71 \pm 1.18\,\mu g\,kg^{-1}$. The PART method detected a slightly higher level ($6.03 \pm 1.51\,\mu g\,kg^{-1}$) and another two positive samples, with OTA levels above the limit of detection (LOD), but under the limit of quantification (LOQ). In Set II, the highest OTA content using VICAM was assessed in sample 20 ($48.63 \pm 12.16\,\mu g\,kg^{-1}$) and the second highest in sample 12 ($31.43 \pm 7.86\,\mu g\,kg^{-1}$). Based on results obtained using the PART method, the order was the opposite, as the highest content was found in sample 12 ($44.74 \pm 11.19\,\mu g\,kg^{-1}$) and the second highest in sample 20 ($34.40 \pm 8.6\,\mu g\,kg^{-1}$). In the second set of samples, the VICAM method detected nine positive samples. The PART method confirmed this finding in all these samples and identified another six positive ones, with two having OTA in concentrations of $0.26 \pm 0.07\,\mu g\,kg^{-1}$ and $0.58 \pm 0.15\,\mu g\,kg^{-1}$ and four being between the LOD and the LOQ. Statistical analysis for the 24 sample measurements (Set II samples and two RMs), *t*-test ($p = 0.20$) and correlation analysis showed no significant bias between the VICAM and PART methods and good correlation between measured values, suggesting a good agreement between both methods. A comparison of inter-method differences and measured values show no trend in absolute differences.

OTA and CIT occurrence in raw cereals

OTA and CIT are naturally produced by fungi, which can contaminate cereals, beans, nuts, spices, and coffee during harvest, transport, processing, and storage. Studies in the field have found OTA usually – but not always – to be absent in cereals at harvest (Elmholt 2003; Molinié 2004). If it is detected in samples immediately after harvest, this is generally due to insufficient care for the sample between its collection

and analysis (Scudamore 2005) or to unclean harvesting machines. Results of the current study correspond with these findings. Among ten wheat samples (Set I) that were analysed shortly after harvest (a maximum after 4 months) and not stored, there was only one sample with an increased OTA content ($4.71 \pm 1.18\,\mu g\,kg^{-1}$) and it was also the only one that was CIT positive, but with a low CIT content not exceeding the LOQ of the method ($1.50\,\mu g\,kg^{-1}$). Subsequent analyses of the content of admixtures and impurities revealed that this sample contained a high amount of weed seeds (included in category 5 Miscellaneous impurities, according to Commission Regulation (EC) No. 824/2000; European Commission 2000). Samples of Set I were chosen from the harvest 2006, which was characterised by unusually rainy weather between 3 and 10 August 2006 in the whole country, and samples delivered to the laboratory later than 25 August were taken for analysis. Rainy weather during the harvest corresponds to a high content of sprouted grains in these samples, which reached up to 47.6% for sample 4. The only sample with a positive OTA and CIT contents (number 2) from this series was harvested on 3 August and did not contain any sprouted grains as the only one. There could be two possible reasons for positive OTA and CIT contents. Either the high content of weed seeds could have led to a temporary post-harvest increase in wheat grain moisture and/or the sample was harvested during the beginning of the rain and was not dried. The moisture content of the sample upon arrival at the laboratory was at a standard 12.5%, but nearly 4 weeks had passed between harvest and the sample's delivery to the laboratory. It could be supposed that the sample had not been treated appropriately immediately after harvest.

Of Set II, only sample 11, naked barley from an organic farming system intended for the production of wholemeal foods, had not been stored for a longer time. It was positive for OTA content, but the concentration was low ($0.49 \pm 0.12\,\mu g\,kg^{-1}$). CIT content was between the LOD and the LOQ. This sample was taken from a tractor trailer on a farm where it had been temporarily left after harvest for approximately 2 weeks. The other nine barley samples were from the 2007 harvest, i.e. they had been stored approximately one year before analysis. The highest content was found in a barley sample for animal feeding, number 20, at $48.63 \pm 12.16\,\mu g\,kg^{-1}$. For OTA in cereals intended for animal feeding, only guidance values are given – in this case $0.25\,mg\,kg^{-1}$ (European Commission 2006b). This value was not exceeded. This sample also contained a higher amount of CIT ($13.17 \pm 3.29\,\mu g\,kg^{-1}$). A high content of broken grains (18.21% in comparison with the maximum limit of 5%) and also miscellaneous impurities (3.91% in comparison with a maximum limit of 3%) were found in this sample. The second highest OTA content ($31.43 \pm 7.86\,\mu g\,kg^{-1}$) and the highest CIT content ($93.64 \pm 28.09\,\mu g\,kg^{-1}$) were determined in sample 12, the barley offered to a malt house but not accepted due to a high admixtures and impurities content and smell of mould. As it is a raw material for food production, OTA content should meet the maximum limit according to Commission Regulation No. 1881/2006 for unprocessed cereals, $5\,\mu g\,kg^{-1}$. Thus, the limit was exceeded by more than six times. This sample was found to have an above-limit content of miscellaneous impurities (14.35% in comparison with a maximum limit of 3%), which consisted mainly of matter passing through a sieve with apertures of 1.0 mm (i.e. mostly dust). In total, among ten barley samples analysed, six were OTA positive and five exceeded the LOQ. Of twelve wheat samples in Set II, three were OTA positive and two exceeded the LOQ. Sample 21 (bread wheat, 2005 harvest) contained OTA at $4.12 \pm 1.03\,\mu g\,kg^{-1}$. This sample also had a higher content of miscellaneous impurities (5.34%). The second above-LOQ OTA sample was sample 25 (bread wheat), with $OTA = 0.33 \pm 0.08\,\mu g\,kg^{-1}$.

Within the current study, in total 32 raw cereal samples – ten of barley and 22 of wheat – were analysed, of which six barley (60%) and four wheat (18%) samples were OTA positive. Among eight cereal samples with an OTA value above the LOQ, four exceeded the permitted level of 3% for miscellaneous impurities and one of them (barley number 20) simultaneously for broken grains. Four barley samples had been intended for food production, of which three were OTA positive and one sample exceeded the limit of $5\,\mu g\,kg^{-1}$ by more than six times. Among eleven wheat samples for food use, three were OTA positive, while two approached the limit of $5\,\mu g\,kg^{-1}$.

The detected maximum OTA levels for barley ($48.63 \pm 12.16\,\mu g\,kg^{-1}$) and wheat ($4.71 \pm 1.18\,\mu g\,kg^{-1}$) are in a broad range of maximum OTA values for raw cereals as reported by various authors (Rizzo et al. 2002). In the Czech Republic, Malir et al. (2006) reported a maximum value of $37.0\,\mu g\,kg^{-1}$. Ten samples in their survey were positive (above an LOD of $0.4\,\mu g\,kg^{-1}$), comprising 8.8% of all 114 samples examined. In our survey, we detected ten positive samples (above an LOD of $0.25\,\mu g\,kg^{-1}$) among 32 examined, i.e. 31%, but the choice of the samples was not fully random and the extent of the sample set was small. If we had evaluated the results obtained using a PART method with an LOD of $0.05\,\mu g\,kg^{-1}$, 18 samples would have been OTA positive, i.e. 56%. The reported percentage of OTA-positive cereal samples varies largely. According to a survey by Pittet (1998), for example, it ranges from 4% to 88%. These levels are influenced by the LOD of the analytical method used, as well as by the number of analysed samples and sampling technique. It is also important whether

only freshly harvested samples are analysed or a survey involves samples that have been stored for some time.

Citrinin is known to be a contaminant in a variety of cereals (Abramson 1997) and could have the same producer (*P. verrucosum*) as OTA, but very few studies have included parallel analyses of both toxins. Vrabcheva et al. (2000) monitored incidences of OTA and CIT in cereal samples from villages where Balkan endemic nephropathy had occurred. Maximum OTA levels in their survey were found for oats (140 and 85 $\mu g\,kg^{-1}$) and for wheat (39 and 31 $\mu g\,kg^{-1}$) using enzyme-linked immunoabsorbant assay (ELISA) and high-performance liquid chromatography (HPLC) methods, respectively. In samples simultaneously contaminated with OTA and CIT, CIT levels were two to 200 times higher than those of OTA. A maximum CIT content (420 $\mu g\,kg^{-1}$) was found in wheat having the highest OTA content. The fact that when simultaneous contamination of cereals occurs CIT concentrations often exceed by several times the OTA concentrations is also reported by Scott (1994). This is also confirmed for breakfast cereals by findings of Molinié et al. (2005), who found more OTA-positive than CIT-positive samples. Nevertheless, if CIT were present, its content was higher than that of OTA. In their study, for a sample with a maximum CIT content (42 $\mu g\,kg^{-1}$), OTA content was 4.1 $\mu g\,kg^{-1}$. In the current study, of 32 cereal samples analysed for CIT content, nine were positive and four exceeded the LOQ for CIT (1.5 $\mu g\,kg^{-1}$). Three of these four samples were simultaneously contaminated with OTA above the LOQ and the CIT:OTA content ratios for these samples were 0.27, 2.08 and 2.98 for OTA as analysed by VICAM, and 0.38, 5.10 and 2.09 for OTA as analysed by PART. The maximum CIT content ($93.64 \pm 28.09\ \mu g\,kg^{-1}$) was found in a barley sample with an OTA content measuring 31.43 ± 7.86 and $44.74 \pm 1.18\ \mu g\,kg^{-1}$ as analysed using VICAM and PART, respectively. All samples with CIT values above the LOQ exceeded the permitted level of 3% for miscellaneous impurities and one of them (barley number 20) simultaneously for broken grains. The highest amount of CIT ever reported was found in French wheat for a farm sample at a level of 520 $\mu g\,kg^{-1}$ (Molinié 2004; Pfohl-Leszkowicz et al. 2007).

The results tend to show that an increased amount of admixtures and impurities in a sample can indicate a higher risk of OTA's occurrence. This fact can be associated, for instance, with the content of weed seeds and parts that are harvested together with a cereal crop. This content can increase grain moisture above a critical level after harvest and thus allow OTA production under favourable temperature conditions. Also, dust seemed to be a risk factor in those samples analysed. As reported by Tangni and Pussemier (2006), dust acts as both an OTA and CIT contaminant and inoculum. They recommend taking precautionary measures not only by controlling and maintaining moisture at a reasonable level during storage of the raw grains, but also by paying close attention to cleaning the storage spaces before placing the new harvests there. They concluded that the presence of dust in grains can be considered a threat to exceeding the legal limits. Cereals can also be contaminated during harvest, as *P. verrucosum* can survive in the field and proliferate on soil organic matter (Elmholt and Hestbjerg 1999). Given appropriate environmental conditions, this may also constitute a risk for grain contamination. OTA content in cereals depends on a variety of factors, including the source of *P. verrucosum* infection, whether or not water activity rises above a critical value (Magan and Aldred 2005), temperature, and storage time. Timely cleaning of cereals and reducing the moisture content below a critical limit can prevent increased OTA from appearing.

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References

Abramson D. 1997. Toxicants of the genus *Penicillium*. In: d'Mello JFP, editor. CRC Handbook of plant and fungal toxicants. Boca Raton (FL): CRC Press. p. 303–317.

Abramson D, Hulasare R, White NDG, Jayas DS, Marquardt RR. 1999. Mycotoxin formation in hulless barley during granary storage at 15% and 19% moisture content. J Stored Prod Res. 35:297–305.

Abramson D, Mills JT, Sinha RN. 1990. Mycotoxin production in amber durum wheat stored at 15 and 19% moisture content. Food Addit Contam. 7:617–627.

Anselme M, Tangni EK, Pussemier L, Motte JC, van Hove F, Schneider YJ, van Peteghem C, Larondelle Y. 2006. Comparison of ochratoxin A and deoxynivalenol in organically and conventionally produced beers sold on the Belgian market. Food Addit Contam. 23:910–918.

Arrai M, Hibino T. 1983. Tumorigenicity of citrinin in male F344 rats. Cancer Lett. 17:281–287.

Bédouret S, Molinié A, Dunnigan P, Castegnaro M, Bony M, Thisse M, Le Boulc'h V, Seng JM, Pfohl-Leszkowicz A. 2001. Contribution à l'amélioration de la qualité sanitaire du blé en cours de stockage. suivi de la formation de mycotoxines, Partie 2: Programme de recherche relatif à leur contaminatin par des champignons toxinogènes producteurs d'ochratoxine A. Phytoma, La défense des végétaux. 541:31–37.

Bettina V. 1984. Mycotoxins: Production, isolation, separation, purification. Amsterdam (the Netherlands): Elsevier. Citrinin and related substances. p. 217–236.

Birzele B, Prange A, Krämer J. 2000. Deoxynivalenol and ochratoxin A in German wheat and changes of level in relation to storage parameters. Food Addit Contam. 17:1027–1035.

Castegnaro M, Tozlovanu M, Wild C, Molinié A, Sylla A, Pfohl-Leszkowicz A. 2006. Advantages and drawbacks of immunoaffinity columns in analysis of mycotoxins in food. Mol Nutr Food Res. 50:480–481.

Chandelier A, Michelet JY, Tangni EK, Baert K, Moons E, Vinkx C. 2004. An overview on toxigenic fungi and mycotoxins in Europe. Dordrecht (the Netherlands): Kluwer. Mycotoxins survey in Belgium and toxigenic Fusarium Belgian wheat. p. 11–32.

Ciegler A, Fennell DI, Sansing GA, Detroy RW, Bennett GA. 1973. Mycotoxin producing strains of *Penicillium viridicatum*: classification into subgroups. Appl Microbiol. 26:271–278.

Conkova E, Laciakova A, Styriak I, Czerwiecki L, Wilczinska G. 2006. Fungal contamination and the levels of mycotoxins (DON and OTA) in cereal samples from Poland and East Slovakia. Czech J Food Sci. 24:33–40.

Creppy EE, Kane A, Dirheimer G, Lafarge-Frayssinet C, Mousset S, Frayssinet C. 1985. Genotoxicity of ochratoxin A in mice: DNA single-strand break evaluation in spleen, liver and kidney. Toxicol Lett. 28:29–35.

DeGroene EM, Hassing MJ, Maarten IGAM, Blom J, Semen W, Fink-Gremmels J, JeanHorbach G. 1996. Development of human cytochrome P450-expressing cell lines: application in mutagenicity testing of ochratoxin A. Cancer Res. 56:299–304.

Elmholt S. 2003. Ecology of the ochratoxin A producing *Penicillium verrucosum*: occurrence in field soil and grain with special attention to farming system and on-farm drying practices. Biol Agric Horticult. 20:311–337.

Elmholt S, Hestbjerg H. 1999. Field ecology of the ochratoxin A-producing *Penicillium verrucosum*: survival and resource colonisation in soil. Mycopathologia. 147:67–81.

Entwisle AC, Williams AC, Mann PJ, Slack PT, Gilbert J. 2000. Liquid chromatographic method with immunoaffinity column cleanup for determination of ochratoxin A in barley: collaborative study. J AOAC Int. 83:1377–1383.

European Commission. 2000. Commission Regulation (EC) No. 824/2000 of 19 April 2000 establishing procedures for the taking-over of cereals by intervention agencies and laying down methods of analysis for determining the quality of cereals. Off J Eur Union. L100:31–50.

European Commission. 2006a. Commission Regulation (EC) No. 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. Off J Eur Union 2006. L364:5–24.

European Commission. 2006b. Commission Recommendation 2006/576/EC of 17 August 2006 on the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2 and fumonisins in products intended for animal feeding. Off J Eur Union 2006. L229:7–9.

European Commission. 2006c. Commission Regulation (EC) No. 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. Off J Eur Union 2006. L364:5–24.

Frank HK. 1992. Citrinin. Z Ernahrungswiss. 31:167–172.

Frisvad JC, Viuf BT. 1986. Methods for the mycological examination of food. New York (MY): Plenum. Comparison of direct and dilution plating for detecting *Penicillium viridicatum* in barley containing ochratoxin. p. 45–47.

Harcz P, Tangni EK, Wilmart O, Moons E, van Peteghem C, De Saeger S, Schneider YJ, Larondelle Y, Pussemier L. 2007. Intake of ochratoxin A and deoxynivalenol through beer consumption in Belgium. Food Addit Contam. 24:910–916.

International Agency for Research on Cancer (IARC). 1976. Some naturally occurring substances. Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Humans, Vol. 10. Lyon (France): IARC.

International Agency for Research on Cancer (IARC). 1983. Some food additives, feed additives and naturally occurring substances. Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Humans, Vol. 31. Lyon (France): IARC.

International Agency for Research on Cancer (IARC). 1987. Overall evaluations of carcinogenicity. Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Humans, Suppl. 7. Lyon (France): IARC.

International Agency for Research on Cancer (IARC). 1993. Some naturally occurring substances: Food items and constituents, heterocyclic aromatic amines, and mycotoxins. Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Humans, Vol. 56. Lyon (France): IARC.

Iwahashi H, Kitagawa E, Suzuki Y, Ueda Y, Ishizawa Y, Nobumasa H, Kuboki Y, Hosoda H, Iwahashi Y. 2007. Evaluation of toxicity of the mycotoxin citrinin using yeast ORF DNA microarray and oligo DNA microarray. BMC Genomics. 8:95, doi: 10.1186/1471-2164-8-95.

Joint FAO/WHO Expert Committee on Food Additives (JECFA). 2001. Joint FAO/WHO Expert Committee on Food Additives, 56th Meeting, Geneva, Switzerland, 6–15 February 2001.

Kanizawa M. 1984. Synergistic effect of citrinin on hepatorenal carcinogenesis of ochratoxin A in mice. Develop Food Sci. 7:245–254.

Krogh P. 1976. Advances in veterinary science and comparative medicine. Vol. 20. New York (NY): Academic PressMycotoxic nephropathy. p. 47–170.

Krogh P, Hald B, Pedersen EJ. 1973. Occurrence of ochratoxin A and citrinin in cereals associated with mycotoxic porcine nephropathy. Acta Pathol Microbiol Scand [B] Microbiol Immunol. 816:689–695.

Lebrun S, Fő llmann W. 2002. Detection of ochratoxin A-induced DNA damage in MDCK cells by alkaline single cell gel electrophoresis (comet assay). Arch Toxicol. 75:734–741.

Lee SC, Chu FS. 1984. Enzyme-linked immunosorbent assay of ochratoxin A in wheat. J AOAC Int. 67:45–49.

Lepom P. 1986. Simultaneous determination of the mycotoxins citrinin and ochratoxin A in wheat and barley by high-performance liquid chromatography. J Chromatogr. 355:335–339.

Levi CP. 1975. Collaborative study of a method for the determination of ochratoxin A in wheat. J AOAC Int. 67:45–49.

Lombaert GA, Pellaers P, Roscoe V, Mankotia M, Nile R, Scott PM. 2003. Mycotoxins in infant cereal foods from the Canadian retail market. Food Addit Contam. 20:494–504.

Lund F, Frisvald JC. 2003. *Penicillium verrucosum* in wheat and barley indicates presence of ochratoxin A. J Appl Microbiol. 95:1117–1123.

Madhyastha SM, Marquardt RR, Frolich AA, Platford G, Abramson D. 1990. Effect of different cereal and oilseed substrates on the growth and production of toxins by *Aspergillus alutaceus* and *Penicillium verrucosum*. J Agr Food Chem. 38:1506–1510.

Magan N, Aldred D. 2005. Conditions of formation of ochratoxin A in drying, transport and in different commodities. Food Addit Contam. Suppl. 1:10–16.

Malir F, Ostry V, Grosse Y, Roubal T, Skarkova J, Ruprich J. 2006. Monitoring the mycotoxins in food and their biomarkers in the Czech Republic. Mol Nutr Food Res. 50:513–518.

Miljkovic A, Pfohl-Leszkowicz A, Dobrota M, Mantle PG. 2002. Comparative responses to mode of oral administration and dose of ochratoxin A or nephrotoxic extract of *Penicillium polonicum* in rats. Exp Toxic Pathol. 54:305–312.

Miller JD. 1996. Food-borne natural carcinogens: issues and priorities. Afr Newslett Occup Hlth Safety. 6(Suppl. 1):22–28.

Molinié A, 2004. Qualité sanitaire des blés en région Midi-Pyrénées: suivi du taux de contamination en mycotoxines de stockage (ochratoxine A et citrinine). Étude des effets biologiques de l'exposition à ces deux toxines. PhD (Thesis) Toulouse (France): Institut National Polytchnique.

Molinié A, Faucet V, Castegnaro M, Pfohl-Leszkowicz A. 2005. Analysis of some breakfast cereals on the French market for their contents of ochratoxin A, citrinin and fumonisin B_1: development of a method for simultaneous extraction of ochratoxin A and citrinin. Food Chem. 923:391–400.

Morgan MRA, McNerney R, Chan HWS. 1983. Enzyme linked immunosorbent assay of ochratoxin A in barley. J AOAC Int. 66:1481–1484.

Nesheim S, Hardin NF, Eppley J. 1973. Analysis of ochratoxins A and B and their esters in barley, using partition and thin-layer chromatography. II Collaborative study. J AOAC Int. 56:817–821.

Nesheim S, Stack ME, Trucksess RM, Eppley J. 1992. Rapid solvent-efficient method for liquid chromatographic determination of ochratoxin A in corn, barley, and kidney: collaborative study. J AOAC Int. 75:481–488.

Palma N, Cinelli S, Sapora O, Wilson SH, Dogliotti E. 2007. Ochratoxin A-induced mutagenesis in mammalian cells is consistent with the production of oxidative stress. Chem Res Toxicol. 207:1031–1037.

Petkova-Bocharova T, Castegnaro M. 1991. Mycotoxins, endemic nephropathy and urinary tract tumours. No. 115. Lyon (France): IARC Press. Ochratoxin A in human blood in relation to Balkan endemic nephropathy and urinary tract tumours in Bulgaria; p. 135–137.

Pfohl-Leszkowicz A, Chakor K, Creppy EE, Dirheimer G. 1991. Mycotoxins, endemic nephropathy and urinary tract tumours. No. 115. Lyon (France): IARC Press. DNA adducts formation in mice treated with ochratoxin A; p. 245–253.

Pfohl-Leszkowicz A, Grosse Y, Obrecht S, Kane A, Castegnaro M, Creppy EE, Dirheimer G. 1993. Preponderance of DNA-adducts in kidney after ochratoxin exposure. In: Human ochratoxicosis and its pathologies. Colloque INSERM, John Libbey Eurotext, Montrouge (France): p. 199–207.

Pfohl-Leszkowicz A, Manderville R. 2007. Review on ochratoxin A: an overview on toxicity and carcinogenicity in animals and humans. Mol Nutr Food Res. 51:61–99.

Pfohl-Leszkowicz A, Molinié A, Castegnaro M. 2006. Underestimation of fumonisin B1 and ochratoxin A, from complex matrices by use of immunoaffinity columns. In: Njapau H, Trujillio S, van Egmond HP, Park DL editors. Mycotoxins and phycotoxins. Proceeding of the 11th International IUPAC Symposium on Mycotoxins and Phycotoxins, 17–21 May 2004, Bethesda, MD, USA. Wageningen (the Netherlands): Wageningen Academic Publishers; p. 83–89.

Pfohl-Leszkowicz A, Molinié A, Tozlovanu M, Manderville RA. 2008. Combined toxic effects of ochratoxin A and citrinin, in vitro and in vivo. In: Siantar DP, Trucksess MW, Scott PM, Herman EM, editors. : Food contaminants; Mycotoxins & food allergen. Washington, DC (USA): ACS Symposium series 1001. p. 56–80.

Pfohl-Leszkowicz A, Petkova-Bocharova T, Chernozemsky IN, Castegnaro M. 2002. Balkan endemic nephropathy and associated urinary tract tumours: a review on aetiological causes and the potential role of mycotoxins. Food Addit Contam. 19:282–302.

Pfohl-Leszkowicz A, Tozlovanu M, Manderville R, Peraica M, Castegnaro M, Stefanovic V. 2007. New molecular and field evidences for the implication of mycotoxins but not aristolochic acid in human nephropathy and urinary tract tumor. Mol Nutr Food Res. 51:131–146.

Pitt JI. 1987. *Penicillium viridicatum*, *Penicillium verrucosum*, and production of ochratoxin A. Appl Environ Microbiol. 53:266–269.

Pittet A. 1998. Natural occurrence of mycotoxins in food and feeds – an updated review. Rev Medec Veterin. 149:479–492.

Rizzo A, Eskola M, Atroshi F. 2002. Ochratoxin A in cereals, foodstuffs and human plasma. Eur J Plant Pathol. 108:631–637.

Scott PM. 1977. Mycotoxic fungi, mycotoxins, mycotoxicoses. An encyclopedic handbook. Vol. 1. New York (NY): Marcel Dekker. Penicillium mycotoxins. p. 283–356.

Scott PM. 1994. Mycotoxins in grain. St. Paul (MN): Eagan. *Penicillium* and *Aspergillus* toxins; p. 261–285.

Scott PM, van Walbeek W, Kennedy B, Anyeti D. 1972. Mycotoxins (ochratoxin A, citrinin, and sterigmatocystin) and toxigenic fungi in grains and other agricultural products. J Agric Food Chem. 20:1103–1109.

Scudamore KA. 2005. Prevention of ochratoxin A in commodities and likely effects of processing

fractination and animal feeds. Food Addit Contam. Suppl. 1:14–25.

Segvic-Klaric M, Zeljezic D, Domijan AM, Peraica M, Pepeljnjak S. 2007. Cytotoxicity, genotoxicity and apoptosis induced by ochratoxin A and citrinin in porcine kidney PK15 cells: effects of single and combined mycotoxins. Toxicol Lett. 172(Suppl. 1):56.

Shephard GS, Berthiller F, Dorner J, Krska R, Lombaert GA, Malone B, Maragos C, Sabino M, Solfrizzo M, Trucksess MW, van Egmond HP, Whitaker TB. 2009. Developments in mycotoxin analysis: an update for 2007–2008. World Mycotox J. 2:3–21.

Tangni EK, Ponchaut S, Maudoux M, Rozenberg R, Larondelle Y. 2002. Ochratoxin A in domestic and imported beers in Belgium: occurrence and exposure assessment. Food Addit Contam. 19:181–189.

Tangni EK, Pussemier L. 2006. Ochratoxin A and citrinin loads in stored wheat grains: impact of grain dust and possible prediction using ergosterol measurement. Food Addit Contam. 23:181–189.

VICAM. 2005. OchraTest^{TM} and OchraTest^{TM}WB HPLC instruction manual. Effective date: November 1, 2005. Instruction Manual # GN-MC9546-0. Watertown, MA (USA): VICAM.

Visconti A, De Girolamo A. 2005. Fitness for purpose – Ochratoxin A analytical developments. Food Addit Contam. Suppl. 1:37–44.

Vrabcheva T, Usleber E, Dietrich R, Märtlbauer E. 2000. Co-occurrence of ochratoxin A and citrinin in cereals from Bulgarian villages with a history of Balkan endemic nephropathy. Agric Food Chem. 486:2483–2488.

Wolff J. 2000. Ochratoxin in cereal and cereal products. Arch Lebensmittelhygiene. 51:81–88.