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OCHRATOXIN A (addendum)

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1. EXPLANATION

Ochratoxin A was first evaluated by the Committee at its thirty-seventh meeting (Annex 1, reference 94). The key adverse effects noted involved toxicity to the kidney. The Committee established a provisional tolerable weekly intake (PTWI) of 112 ng/kg body weight (bw), on the basis of deterioration of renal function in pigs, for which the lowest-observed-effect level (LOEL) was 8 µg/kg bw per day, and application of a safety factor of 500. At that time, the Committee recommended that further studies be conducted to elucidate the role of ochratoxin A in causing nephropathy in pigs, the mode of action of ochratoxin A as a kidney carcinogen in rodents and the possible role of ochratoxin A in human disease.

Ochratoxin A was re-evaluated by the Committee at its forty-fourth meeting (Annex 1, reference 116), when it considered toxicological data that had become available since the previous evaluation, including epidemiological studies of nephropathy, genotoxicity studies and studies on experimental nephrotoxicity. At that meeting, the Committee reconfirmed the PTWI, rounding it to 100 ng/kg bw, and reiterated its request for further studies on ochratoxin A.

Ochratoxin A was again evaluated by the Committee at its fifty-sixth meeting (Annex 1, reference 152), when it was noted that the adverse effect at the lowest effective dose in several mammalian species was nephrotoxicity and that this was likely to be true in humans as well. Although an association between the intake of ochratoxin A and nephropathy in humans had been postulated, causality had not been established. Concerning carcinogenicity, the Committee concluded that the new toxicological data available since the previous evaluation raised further questions about the mode of action of ochratoxin A. Both genotoxic and non-genotoxic modes of action had been proposed. The Committee noted that further studies to address these issues were in progress and retained the previously established PTWI of 100 ng/kg bw, pending the results of these studies.

New data from surveys of food commodities for contamination with ochratoxin A were also considered, and intakes were estimated for various countries and regions of the world. From estimates based mainly on European data, the Committee noted that the intake of ochratoxin A by 95th percentile consumers of cereals may approach the PTWI from this source alone. Given the distribution of contamination of cereals with ochratoxin A, the Committee concluded that application of a maximum limit (ML) of 5 or 20 µg/kg would make no significant difference to the average intake. Efforts were needed to ensure that intakes of ochratoxin A did not exceed the PTWI, and the Committee considered that this could

best be achieved by lowering overall contamination by appropriate agricultural, storage and processing practices.

At the thirty-eighth session of the Codex Committee on Food Additives and Contaminants (CCFAC) (Codex Alimentarius Commission, 2006), the Committee was again asked to re-evaluate ochratoxin A, considering all the data available on toxicology and exposure assessment, particularly data from developing countries, including the impact of different MLs for cereals (5 or 20 µg/kg) and effects of processing on residual levels in foods.

For this evaluation, the Committee considered new toxicological studies that had become available since the last evaluation; these included further studies on developmental toxicity, neurotoxicity, immunotoxicity, nephrotoxicity and genotoxicity and studies on the mode of action of ochratoxin A in the kidney. The Committee also considered the opinion on ochratoxin A in human food published by the European Food Safety Authority (EFSA) in 2006 (European Food Safety Authority, 2006). New data on analytical methods, sampling protocols and the effects of processing were also considered, together with methods of prevention and control and levels and patterns of food contamination. A new dietary exposure assessment was conducted, and the impact of different MLs for cereals was considered.

2. BIOLOGICAL DATA

In this addendum, relevant findings for risk assessment published since the Committee's last evaluation (Annex 1, reference 153) are described. In addition, for aspects critical to the current re-evaluation (in particular, the mode of action of ochratoxin A as a kidney carcinogen), some of the data evaluated previously by this Committee are briefly summarized in order to set the context for the more recent information.

2.1 Biochemical aspects

2.1.1 Absorption, distribution, metabolism and excretion

The absorption, distribution, metabolism and excretion of ochratoxin A have been summarized previously by the Committee as follows (Annex 1, reference 153). Ochratoxin A is efficiently absorbed from the gastrointestinal tract, mainly in the small intestine. Information from a number of species shows that it is distributed via the blood, mainly to the kidneys, with lower concentrations being found in liver, muscle and fat. Transfer to milk has been demonstrated in rats, rabbits and humans, but little is transferred to the milk of ruminants, owing to hydrolysis of ochratoxin A into phenylalanine and ochratoxin *alpha* by the rumenal microflora. The major metabolite of ochratoxin A in all species examined is ochratoxin *alpha*, formed by hydrolysis of the peptide bond. Ochratoxin *alpha* and minor hydroxylated metabolites that have been identified are all reported to be less toxic than ochratoxin A itself. Ochratoxin A is excreted in urine and faeces, and the relative contribution of each of these routes in different species is influenced by the extent of the

enterohepatic recirculation of ochratoxin A and its binding to serum protein. These factors are also important in the determination of the serum half-life of ochratoxin A, which varies widely among species. It has a long half-life in some non-ruminant mammals (e.g. 1–1.5 days in mice, 2–5 days in rats, 3–5 days in pigs, 21 days in one macaque and 35 days in a human volunteer).

Additional information published since the Committee's previous evaluation is described below.

The metabolism of ochratoxin A was studied in cultured rat and human primary hepatocytes incubated with non-cytotoxic concentrations of [^3H]ochratoxin A ranging from 10^7 to 10^5 mol/l for 8 h. In rat hepatocytes, ochratoxin A was metabolized to small amounts of three products. In addition to 4-hydroxy-ochratoxin A, which is a known product of ochratoxin A biotransformation, two novel metabolites were detected and tentatively identified as hexose and pentose conjugates of ochratoxin A. In vitro induction with 3-methylcholanthrene increased the formation of 4-hydroxy-ochratoxin A but did not alter the formation of the conjugated metabolites (Gross-Steinmeyer et al., 2002).

A study was performed to investigate the biotransformation and toxicokinetics of orally administered ochratoxin A in rats. Groups of 18 male and 18 female F344 rats were given ochratoxin A at a single oral gavage dose of 0.5 mg/kg bw, in corn oil. Groups of three animals were sacrificed at intervals between 24 and 1344 h after ochratoxin A administration, and concentrations of ochratoxin A and its metabolites in urine, faeces, blood, liver and kidney were determined. There was efficient absorption of ochratoxin A from the gastrointestinal tract and only slow elimination and little biotransformation. Recovery of unchanged ochratoxin A in urine as a percentage of the dose was only 2.1% in males and 5.2% in females within 96 h. The majority of the dose was absorbed and still present in the systemic circulation or tissues at 96 h. In faeces, 5.5% and 1.5% of the dose were recovered in males and females, respectively, within 96 h. The major metabolite detected was ochratoxin *alpha*. Low concentrations of ochratoxin A–glucosides (pentose and hexose conjugates) were also reported for the first time in urine. The maximal blood levels of ochratoxin A were observed between 24 and 48 h after administration and were approximately 4.6 $\mu\text{mol/l}$ in males and 6.0 $\mu\text{mol/l}$ in females. Elimination of ochratoxin A from blood followed first-order kinetics, with a half-life of approximately 230 h. In liver of both male and female rats, concentrations of ochratoxin A were less than 12 pmol/g tissue, with a maximum at 24 h after administration. In contrast, ochratoxin A accumulated in the kidneys, reaching a concentration of 480 pmol/g tissue in males 24 h after administration of ochratoxin A. Ochratoxin *alpha* was not detected in liver and kidney tissue, and the concentrations in blood were low (10–15 nmol/l). The authors concluded that the high concentrations of ochratoxin A in kidneys of male rats may, in part, explain the organ- and sex-specific toxicity of ochratoxin A (Zepnik et al., 2003).

The EFSA opinion (European Food Safety Authority, 2006) discusses the hypothesis that specific transporters may be involved in the cellular uptake of ochratoxin A into the kidney, where it accumulates. It has been suggested that the marked differences in the relative sensitivity of individual species and sexes towards

ochratoxin A might be due to variations in the transport mechanisms and cellular uptake in renal cells. In vitro data with cell cultures indicate that ochratoxin A is a substrate for the family of organic anion transporter (OAT) proteins. In humans, the best characterized of these transporters, OAT1, encoded by the *SLC22A* gene family, has wide substrate specificity. The rodent equivalents of the human proteins are also encoded by members of the *SLC22A* gene family. Expression of OAT proteins in the kidney shows sex and species differences (Russel et al., 2002). Support for the hypothesis of an involvement of transporters in the observed sex and species differences in ochratoxin A-mediated toxicity is also to be found in the observations of Buist (2002) and Buist & Klaassen (2003), who demonstrated large sex-, age- and species-dependent variations in the expression levels of a number of OATs (Dietrich et al., 2005). Ochratoxin A is also a substrate for transporters of the adenosine triphosphate (ATP)-dependent transporter family, which excrete compounds out of cells (Schrickx et al., 2005). These transporters are assumed to play a role in the excretion of ochratoxin A-glucuronides by the kidneys.

Additional information on the toxicokinetics of ochratoxin A in primates is also available. Three female vervet monkeys, *Cercopithecus aethiops*, were each given a single intravenous dose of 0.8, 1.5 or 2 mg ochratoxin A/kg bw. Blood and urine samples were collected over a period of 21 days. The results suggested that the clearance of ochratoxin A from plasma followed a two-compartment model. The elimination half-life of ochratoxin A in the monkeys was 19–21 days, and the average total body clearance was 0.22 ml/h per kilogram body weight. The mean apparent volumes of distribution of the central compartment and the peripheral compartment were of similar value (59 ml/kg) (Stander et al., 2001).

The toxicokinetic profile of ochratoxin A was studied in one human volunteer following ingestion of 395 ng ³H-labelled ochratoxin A (0.14 MBq). The data suggested a two-compartment open model. This two-compartment model consisted of a fast elimination and distribution phase (half-life about 20 h) followed by a slow elimination phase (renal clearance about 0.11 ml/min), with a calculated plasma half-life of 35 days. In addition, the intraindividual fluctuation of plasma levels of ochratoxin A was investigated in eight volunteers over a period of 2 months. The plasma concentrations determined ranged between 0.2 and 0.9 ng ochratoxin A/ml. The plasma levels in some individuals remained nearly constant over time, whereas others varied considerably (e.g. increase of 0.4 ng/ml within 3 days, decrease of 0.3 ng/ml within 5 days) during the observation period. The authors calculated a renal clearance varying between 0.093 and 0.109 ml/min (approximately 0.13 litres/day), indicating that even a non-regular exposure (consumption of contaminated foods once a week or once a month) can result in persistent blood levels (Studer-Rohr et al., 2000).

The major analyte in human blood serum was the parent compound, and only small concentrations of ochratoxin A metabolites and/or conjugates could be measured. In contrast, analysis of urine samples indicated that only about 50% of the radioactivity in the urine was parent ochratoxin A, suggesting the presence of ochratoxin A metabolites (particularly ochratoxin *alpha*) and/or ochratoxin A-glucuronic acid conjugates (Studer-Rohr et al., 2000).

2.2 Toxicological studies

2.2.1 Short-term studies of toxicity

The short-term toxicity of ochratoxin A has been extensively reviewed previously by this Committee (Annex 1, reference 153) and by EFSA (European Food Safety Authority, 2006). In summary, the kidney is the major target organ for the adverse effects of ochratoxin A, and short-term toxicity studies in mice, rats, dogs and pigs have shown dose-dependent and time-dependent development of progressive nephropathy. There are significant sex and species differences in sensitivity of the kidney to the toxic effects of ochratoxin A. Most of the recent short-term studies on ochratoxin A-induced kidney toxicity have focused on the possible mechanism(s) for its toxic effects, and these are described below in section 2.2.5.

(a) Rats

Male F344/N rats were treated with ochratoxin A orally by gavage at doses of 0, 21, 70 or 210 µg/kg bw per day for 14, 28 or 90 days, 5 days/week. This treatment regimen corresponded to that used in the 2-year bioassay conducted by the United States National Toxicology Program (NTP) (National Toxicology Program, 1989), which established the renal carcinogenicity of low-dose ochratoxin A in rats. Cell proliferation in the renal cortex and the outer stripe of the outer medulla was determined. Histopathological examination showed renal changes in mid- and high-dose animals involving single cell death and prominent nuclear enlargement within the straight segment of the proximal tubules of the outer stripe of the outer medulla, from which ochratoxin A-induced tumours are known to arise. At doses of 70 and 210 µg/kg bw per day, there was a marked dose- and time-dependent increase in renal cell proliferation, extending from the medullary rays into the outer stripe of the outer medulla. No effects were evident in the kidneys of low-dose animals or in the liver. The no-observed-effect level (NOEL) in this study was 21 µg/kg bw per day, which correlates with the dose in the NTP 2-year bioassay that did not produce renal tumours. The apparent correlation between enhanced cell turnover and tumour formation induced by ochratoxin A indicates that stimulation of cell proliferation may play an important role in ochratoxin A carcinogenicity (Rached et al., 2007).

There have been several reports on ochratoxin A-induced apoptosis in the kidney. In rats given ochratoxin A intraperitoneally at doses of 0.25, 0.50 or 1.00 mg/kg bw, 3 times a week for 4 weeks, time- and dose-related increases in apoptosis in the kidneys were observed (Domijan et al., 2004).

Intraperitoneal administration of 120 µg ochratoxin A/kg bw per day to Wistar rats, for 10, 30 or 60 days, produced oxidative stress and dose/time-related apoptosis in both proximal and distal epithelial kidney cells. Ochratoxin A concentrations in the kidneys were proportional to the time of exposure and amounted to 547, 753 and 930 ng/g kidney tissue after 10, 30 and 60 days, respectively. Oxidative stress was evident from increased malondialdehyde formation in the kidney cells (Petrik et al., 2003).

Ochratoxin A was administered orally by gavage to groups of three male Fischer 344 rats at dose levels of 0, 0.25, 0.5, 1 or 2 mg/kg bw per day for 2 weeks. The typical pathological changes, including apoptosis, were seen in the kidneys at all dose levels, with a clear increase in severity with higher doses. A dose-dependent increase in the expression of proliferating cell nuclear antigen, indicative of cell proliferation, was observed in kidneys, but not in livers, of treated animals. The most prominent change in the composition of urine induced by ochratoxin A consisted of a major increase in the excretion of trimethylamine *N*-oxide. This pattern was said to be different from the typical changes observed with other proximal tubular toxins and suggests that a unique mechanism may be involved in ochratoxin A nephrotoxicity and carcinogenicity (Mally et al., 2005a).

(b) *Rabbits*

Ochratoxin A was fed in the diet to groups of four New Zealand White rabbits, starting at 6–8 weeks of age, at a concentration of 0 or 0.75 mg/kg of feed for 60 days, as part of an investigation into the effects of co-administration of ochratoxin A with another mycotoxin, citrinin. Ultrastructural changes in the epithelial cells of the proximal convoluted tubule in the kidney were evaluated. Degenerative and necrotic changes were observed, especially in the mitochondria, together with loss of the brush border, atrophy of microvilli, cytoplasmic vacuolation with loss of organelles, degenerating nucleus and loss of nucleolus (Kumar et al., 2007).

(c) *Pigs*

As described in the previous evaluation of ochratoxin A by this Committee (Annex 1, reference 153), the pig appears to be the most sensitive species with respect to the nephrotoxic effects of ochratoxin A, and the LOEL in pigs for renal effects is the basis of the PTWI.

Groups of three male and three female pigs were exposed to diets contaminated with strains of *Aspergillus ochraceus* producing ochratoxin A and penicillic acid and containing 90, 130 or 180 µg ochratoxin A/kg of diet. Two pigs from each group were examined after 3 months. Microscopic lesions, as well as changes in various haematological and biochemical parameters, were observed in all groups. For an additional 2 months, the ochratoxin A concentrations in the diets were raised to 130, 305 or 790 µg/kg. Histological examination (two pigs per group) at the end of the exposure period showed degenerative changes affecting the epithelial cells of the proximal tubules, which predominated at the initial stage, and proliferative changes in the interstitium, which predominated at the later stage of the disease (Stoev et al., 2001). Subsequently, Stoev et al. (2002) reported only

mild nephropathy in three male and three female pigs given a diet containing ochratoxin A at a concentration of 800 µg/kg for 1 year. Histological examination showed two types of changes: degenerative changes affecting epithelial cells in some proximal tubules of pigs after 6 months, and proliferative changes in the interstitium, which predominated after 1 year of exposure to ochratoxin A.

2.2.2 Long-term studies of toxicity and carcinogenicity

The long-term toxicity and carcinogenicity of ochratoxin A have been extensively reviewed previously by this Committee (Annex 1, reference 153) and by EFSA (European Food Safety Authority, 2006). Ochratoxin A produces renal tumours in mice and rats, and, as is the case for short-term toxicity to the kidney, there are marked sex and species differences. Male animals are more sensitive than females, and rats are considerably more sensitive than mice. The Committee previously noted that the long-term effects in rats were preceded by evidence of renal toxicity in 16-day and 13-week studies and that it was unclear whether the malignancy and aggressive nature of the tumours were a secure indication that the mechanism of induction is via deoxyribonucleic acid (DNA) reactivity (Annex 1, reference 153).

The several hypotheses on the mode of action of ochratoxin A as a carcinogen and the related evidence have been discussed elsewhere (see, for example, reviews by [Fink-Gremmels, 2005](#); [Walker & Larsen, 2005](#); [Rached et al., 2007](#)). These hypotheses will be discussed in section 9, in light of the additional evidence obtained since the Committee's previous review in 2001.

The key features of the carcinogenicity bioassays in mice and rats conducted by the NTP, reviewed previously by this Committee, together with information from these and other related NTP studies concerning the occurrence of karyomegaly as an early pathological indicator of renal toxicity, are summarized below. It should be noted that for male rats, the LOELs and NOELs for karyomegaly are similar to those for tumour formation (Table 1).

Table 1. LOELs and NOELs for karyomegaly and carcinogenicity of ochratoxin A in male mice and rats^a

Species	Effect	Study duration	LOEL (µg/kg bw per day)	NOEL (µg/kg bw per day)
Mouse (male) ^b	Kidney tumours	2 years	4400	130
Rat (male) ^c	Karyomegaly of proximal tubule cells	90 days	62.5	Not established
		9 and 15 months	70	21
	Kidney tumours	2 years	70	21

^a From National Toxicology Program (1989).

^b Ochratoxin A administered in the diet.

^c Ochratoxin A administered by gavage 5 days/week.

Some new data on the carcinogenicity of ochratoxin A have also been presented in the final report of the Ochratoxin A Risk Assessment project, funded by the European Commission (2004). Fischer 344 rats were given ochratoxin A in the feed at a concentration giving intakes equivalent to approximately 300 µg/kg bw per day. A statistical comparison of the total carcinoma incidence from this exposure with that reported in the NTP Fischer 344 rat study (National Toxicology Program, 1989), in which gavage administration had been used, suggested that ochratoxin A is significantly less potent when given in feed than when given by oral gavage. However, the Committee noted that EFSA considered it unsuitable as a basis for a quantitative risk assessment because there was only one dose level in the feed (European Food Safety Authority, 2006), and further details of the study were lacking (Mantle et al., 2005).

In order to provide additional information for the risk assessment, the Committee performed benchmark dose (BMD) modelling using the carcinogenicity data on ochratoxin A from the rat bioassay performed by the NTP (National Toxicology Program, 1989). The occurrence of combined adenomas and carcinomas in the kidneys of the male rats (Table 2), as the most sensitive sex and species for kidney carcinogenicity of ochratoxin A, was considered the most appropriate data for modelling.

The BMD approach has been put forward as an alternative to the no-observed-adverse-effect level (NOAEL) and lowest-observed-adverse-effect level (LOAEL) approach for health effects because it provides a more quantitative alternative point of departure for the first step in the dose-response assessment (International Programme on Chemical Safety, in press). The BMD approach is based on a mathematical model being fitted to the experimental data within the observable range and estimates the dose that causes a low but measurable response (the benchmark response) typically chosen at a 5% or 10% incidence above the control. The BMD lower limit (BMDL) refers to the corresponding lower limit of a one-sided 95% confidence interval on the BMD. Using the lower bound takes into account the uncertainty inherent in a given study and assures (with 95% confidence) that the chosen benchmark response is not exceeded.

Table 2. Renal tumours and karyomegaly in male rats exposed to ochratoxin A^a

Ochratoxin A dose (µg/kg bw per day) ^b	Adenomas	Carcinomas	Adenomas and carcinomas	Karyomegaly
0	1/50	0/50	1/50	0/50
21	1/50	0/50	1/50	1/50
70	6/51	16/50	20/51	51/51
210	10/50	30/50	36/50	50/50

^a From National Toxicology Program (1989).

^b Administered by gavage on 5 days/week for 2 years.

The United States Environmental Protection Agency BMD software version 1.4.1 was used (United States Environmental Protection Agency, 2007) for modelling the kidney tumour dose–response in the male rats. For carcinogenicity data, a number of models are available in the BMD software, and model fitting, determination of goodness of fit and comparing models to decide which one to use for obtaining the BMDL are outlined. The following dose–response models were fitted to the dose–incidence data:

- gamma multihit model;
- log-logistic model;
- multistage model;
- probit model;
- log probit model;
- quantal linear model;
- Weibull model.

The BMD₁₀ and BMDL₁₀ values, for an extra 10% increase in incidence of tumours compared with the background incidence in controls, were estimated by performing 250 iterations.

The acceptability of a model can be based on several criteria. Some of the models are nested models (i.e. they are related to each other such that by leaving out a parameter, one model reduces to the other; this holds for the one-, two- and three-stage models). The fit should not be significantly worse (using the likelihood ratio test) than the fit provided by the “full” model. The full model is the model that does not assume any dose–response function (its parameters are simply the frequencies per dose level) (Filipsson et al., 2003).

While the likelihood ratio test can be applied only to nested models, the Akaike information criterion (AIC) (Akaike, 1974; Bozdogan, 1987) has been proposed as an approximate criterion for comparing the fits of non-nested models (Filipsson et al., 2003).

In addition, the BMD software provides statistics for the goodness of the fit. The lower the chi-square value, the better the fit, and the calculated *p*-value should be significantly larger than 0.1, which in this case was chosen to represent a rejection level (Filipsson et al., 2003).

The BMD₁₀ and BMDL₁₀ values, as well as the associated statistics, for the models used are presented in [Table 3](#).

The calculated BMD₁₀ values ranged from 18 to 33 µg ochratoxin A/kg bw per day, with the most reliable values being around 30 µg ochratoxin A/kg bw per day. The BMDL₁₀ values ranged from the lowest value of 15 µg/kg bw per day up to 25 µg/kg bw per day for the model providing the best fit. Thus, the BMDL₁₀ does not provide a lower point of departure, compared with the LOEL of 8 µg/kg bw per day for minimal renal toxicity changes in the pig, for establishing the PTWI.

Table 3. BMD₁₀ and BMDL₁₀ calculations based on total kidney tumour incidences in male F344 rats from the NTP study^a

Model	Log (likelihood)	p-value	AIC	Chi- square	P-value	Accept	BMD ₁₀ (µg/kg bw per day ^b)	BMDL ₁₀ (µg/kg bw per day ^b)
Full model	-71.61							
Gamma multi-hit	-76.36	0.02	158.7	4.91	0.03	??	30	18
Log-logistic	-75.57	0.05	157.1	3.46	0.06	Yes??	32	21
Multistage	-77.29	0.01	160.6	5.96	0.01	??	24	15
Log-probit	-75.05	0.09	156.1	2.64	0.1	Yes	33	25
Quantal- linear	-77.74	0.02	159.5	5.99	0.05	??	18	15
Weibull	-76.68	0.01	159.4	5.27	0.02	??	28	17
Reduced model	-120.77	<0.001						

^a From National Toxicology Program (1989).

^b Ochratoxin A administered by gavage 5 days/week for 2 years.

2.2.3 Genotoxicity

Numerous in vitro and in vivo genotoxicity studies have been conducted on ochratoxin A, most of which were described previously by this Committee (Annex 1, reference 153). Further studies have been conducted since then to try to clarify the mode of action of ochratoxin A as a carcinogen. Since genotoxicity data are a key element in the present evaluation, a summary of all the relevant data is presented in Table 4.

(a) DNA adducts

The question of whether ochratoxin A can form DNA adducts in the kidney has been intensively studied because of its potential relevance to a genotoxic mode of action for ochratoxin A as a kidney carcinogen. The information on the earlier and more recent studies has been reviewed by several groups (Annex 1, reference 153; Mally & Dekant, 2005; Pfohl-Leszkowicz & Castegnaro, 2005; Turesky, 2005; European Food Safety Authority, 2006; Pfohl-Leszkowicz & Manderville, 2007).

At the time of the Committee's previous evaluation (Annex 1, reference 153), almost all the available studies in which DNA adducts were detected by ³²P-postlabelling after exposure to ochratoxin A were from one laboratory, and all showed positive results, including in rat and mouse kidney DNA (Pfohl-Leszkowicz et al., 1991, 1993; Grosse et al., 1995, 1997; Castegnaro et al., 1998; Pfohl-Leszkowicz et al., 1998). However, the Committee pointed out that the nonspecific postlabelling technique used may have resulted in adducts that did not

Table 4. Summary of results of genotoxicity tests on ochratoxin A

Test system	Test object	Concentration	Results	Reference
In vitro				
<i>Bacterial assays</i>				
Reverse mutation	<i>Salmonella typhimurium</i> TA98, TA100, TA1535, TA1537 and TA1538	0.4–400 µg/plate	Negative (highly variable TA100 controls, not tested to cytotoxicity)	Kuczuk et al. (1978); Wehner et al. (1978)
Reverse mutation	<i>S. typhimurium</i> TA1538	0.1–500 µg/plate (mixture of ochratoxin A:ochratoxin B)	Positive >100 µg/plate	Kuczuk et al. (1978)
Reverse mutation	<i>S. typhimurium</i> TA100 and TA1538	~200 µg/plate	Negative with mouse and rat liver activation	Bartsch et al. (1980)
Reverse mutation	<i>S. typhimurium</i> TA98, TA100, TA1535, TA1537 and TA1538	50–600 µg/plate	Negative	Bendele et al. (1985)
Reverse mutation	<i>S. typhimurium</i> TA98, TA100, TA1535, TA1537, TA1538, G46, G3076 and D3052	0.1–100 µg/ml	Negative	Bendele et al. (1985)
Reverse mutation	<i>S. typhimurium</i> TA97, TA98, TA100 and TA1535	1–100 µg/plate	Negative with hamster or rat liver activation	National Toxicology Program (1989)
Reverse mutation	<i>S. typhimurium</i> TA98, TA100, TA1535, TA1537 and TA1538		Positive after activation by medium derived from hepatocytes exposed to ochratoxin A	Hennig et al. (1991)
Reverse mutation	<i>S. typhimurium</i> TA98, TA1535 and TA1538 (TA100 and TA102 also tested in preliminary study)	0–1200 µg/plate	Positive in all 3 strains after activation by mouse kidney microsomes, but not after mouse liver microsomes (TA100, TA102 negative)	Obrecht-Pflumio et al. (1999)

Table 4. (contd)

Test system	Test object	Concentration	Results	Reference
Reverse mutation	<i>S. typhimurium</i> TA100 and TA2638	0–200 µg/plate	Negative after activation with rat liver and kidney microsomes	Zepnik et al. (2001)
Reverse mutation	<i>S. typhimurium</i> TA98 and TA100	2.5–50 µg/plate	Negative with HepG2-derived enzyme homogenate (S9 mix)	Ehrlich et al. (2002)
Reverse mutation	<i>S. typhimurium</i> TA98, TA100, TA102, TA104, TA1535 and TA1538	0.01–500 µmol/l	Negative with and without rat liver S9 mix	Föllmann & Lucas (2003)
Gene mutation	<i>Saccharomyces cerevisiae</i> D3	75, 200 µg/plate	Negative	Kuczuk et al. (1978)
Gene mutation	<i>Bacillus subtilis</i> rec	20–100 µg/disc	Negative	Ueno & Kubota (1976)
DNA repair	<i>Escherichia coli</i> , SOS assay	1–2 mg/100 µl	Negative	Auffray & Boutibonnes (1986); Reiss (1986)
DNA repair	<i>E. coli</i> WP2	Gradient plate, not stated	Negative	Bendele et al. (1985)
<i>Mammalian cells</i>				
Gene mutation	C3H mouse mammary cells	5–10 µg/ml	Negative (10 µg/ml cytotoxic)	Umeda et al. (1977)
Forward gene mutation	Mouse lymphoma cells, <i>tk</i> locus	0.1–13 µg/ml	Negative (>12 µg/ml cytotoxic)	Bendele et al. (1985)
Forward gene mutation	Chinese hamster V79 fibroblasts, <i>HPRT</i>	0.1–100 µmol/l	Negative with and without rat liver S9 mix	Föllmann & Lucas (2003)
Gene mutation (<i>lacZ</i> on shuttle vector)	NIH/3T3 cells transfected with human cytochrome P450	25 µg/ml	Positive	De Groene et al. (1996)
UDS	ACI rat primary hepatocytes	0.4, 4 µg/ml	Weakly positive at 0.4, cytotoxic at 4 µg/ml	Mori et al. (1984)
UDS	C3H mouse primary hepatocytes	4, 40 µg/ml	Weakly positive at 4, cytotoxic at 40 µg/ml	Mori et al. (1984)

Table 4. (contd)

Test system	Test object	Concentration	Results	Reference
UDS	Fischer 344 rat primary hepatocytes	0.000 025–500 µg/ml (2 lots tested at 15 doses)	Negative (>0.05 µg/ml cytotoxic)	Bendele et al. (1985)
UDS	Rat hepatocytes; porcine urinary bladder epithelial cells	250 nmol/l–1 µmol/l	Positive	Dorrenhaus & Föllmann (1997)
UDS	Cultured human urothelial cells	0.005–0.05 µmol/l	Positive	Flieger et al. (1998)
UDS	Primary human urothelial cells	10–2000 nmol/l	Positive	Dorrenhaus et al. (2000)
DNA strand break, alkaline elution	CHO cells; rat fibroblasts	200 µg/ml	Positive (1.2 strand breaks/109 Da)	Stetina & Votava (1986)
DNA damage	Mouse spleen, phytohaemagglutinin-stimulated	1–10 µg/ml	Positive (concentration-related)	Creppy et al. (1985)
DNA damage, comet assay	Human hepatoma-derived cell line, HepG2	5–30 µg/ml	Positive	Ehrlich et al. (2002)
DNA damage, comet assay	MDCK cells		Positive (concentration-related with and without rat liver S9 mix)	Lebrun & Föllmann (2002)
DNA damage, comet assay	Primary cultured human urothelial cells	100 µmol/l	Variable results, DNA damage was associated with certain polymorphisms of GST enzymes	Lebrun et al. (2006)
DNA damage, comet assay	Mouse fibroblast NIH/3T3 cells transfected with human cytochrome P450 co-expressing human oxidoreductase	10–200 µmol/l	Negative in CYP3A4-expressing cells, positive in CYP2C9-expressing cells	Simaro Doorten et al. (2006)

Table 4. (contd)

Test system	Test object	Concentration	Results	Reference
DNA damage, comet assay	Human kidney proximal tubular cell line, HK-2	50–600 µmol/l	Negative at non-cytotoxic concentrations (with or without rat liver S9 mix) at 3 h; positive at cytotoxic concentrations (≥ 400 µmol/l) at 6 h and at all concentrations with Fpg and Endo III (for detection of oxidative damage)	Arbillaga et al. (2007a, 2007b)
SCE	Human peripheral blood lymphocytes	5–10 µg/ml	Negative (mitotic inhibition at 10 µg/ml)	Cooray (1984)
SCE	CHO cells, 26 h with ochratoxin A	0.5–5 µg/ml	Negative	National Toxicology Program (1989)
SCE	CHO cells, 2 h with ochratoxin A	5–160 µg/ml	Positive (frequency 37% above control, weak dose–response relationship)	National Toxicology Program (1989)
SCE	Human lymphocytes		Positive	Hennig et al. (1991)
SCE	Bovine lymphocytes	0.1–2 µmol/l	Positive (in the presence of reduced cell proliferation and viability and increased apoptosis)	Lioi et al. (2004)
Chromosomal aberration	CHO cells, 8–10 h with ochratoxin A	30–160 µg/ml	Negative	National Toxicology Program (1989)
	2 h with ochratoxin	100–300 µg/ml	Negative	
Chromosomal aberration	Human lymphocytes, 48 h with ochratoxin A	4.5 µg/ml	Positive (4.5- to 5-fold increase)	Manolova et al. (1990)

Table 4. (contd)

Test system	Test object	Concentration	Results	Reference
Chromosomal aberration	Bovine lymphocytes	0.1–2 µmol/l	Positive (in the presence of reduced cell proliferation and viability and increased apoptosis)	Lioi et al. (2004)
Micronucleus formation	Ovine seminal vesicle cell cultures	12–30 µmol/l	Positive ^a	Degen et al. (1997)
Micronucleus formation	Syrian hamster embryo fibroblasts	5–20 µmol/l	Positive ^b	Dopp et al. (1999)
Micronucleus formation	Human hepatoma-derived cell line, HepG2	5–50 µg/ml	Positive (via chromosome breaking effects)	Ehrlich et al. (2002)
In vivo				
Chromosomal aberration	Mouse bone marrow and sperm cells	1 µg/kg bw per day in diet, 15 and 45 days	Positive (ameliorated by 10 mg ascorbic acid/kg bw)	Bose & Sinha (1994)
Chromosomal aberration	Mouse	1 µg/kg bw per day in diet, 14 days	Positive (ameliorated by 130 IU vitamin A/kg bw)	Kumari & Sinha (1994)
Chromosomal aberration	Rat, cultured spleen lymphocytes from animals treated in vivo	250–2000 µg/kg bw by gavage 5 days/week for 2 weeks	Slight increases in aberrations (mainly deletions), but not statistically significant	Mally et al. (2005b)
SCE	Chinese hamster bone marrow	25–400 mg/kg bw by gavage	Negative (>100 mg/kg bw cytotoxic)	Bendele et al. (1985)
DNA damage (single-strand breaks)	BALB/c mouse Spleen 4, 16 and 24 h after treatment	2500 µg/kg bw intraperitoneally	Positive (maximum response at 24 h)	Creppy et al. (1985)

Table 4. (contd)

Test system	Test object	Concentration	Results	Reference
	Kidney 24 and 48 h after treatment		Positive (maximum response at 24 h)	
	Liver 24, 48 and 72 h after treatment		Positive (maximum response at 48 h; recovery at 72 h)	
DNA damage	Wistar rat kidney and liver	290 µg/kg bw by gavage every 48 h for 6 or 12 weeks	Positive, no recovery between treatments	Kane et al. (1986)
DNA damage, comet assay	Rat liver, kidney and spleen	250–2000 µg/kg bw by gavage 5 days/week for 2 weeks	Positive, enhanced by Fpg glycosylase (converts oxidative DNA damage into strand breaks)	Mally et al. (2005b)
DNA damage, comet assay	Rat kidney	500 µg/kg bw per day intraperitoneally for 7, 14 or 21 days	Positive (extent of DNA damage correlated with increasing plasma and kidney ochratoxin A levels over time)	Zeljezic et al. (2006)

CHO, Chinese hamster ovary; CYP, cytochrome P450; Endo III, endonuclease III; Fpg, formamido-pyrimidine-DNA-glycosylase; GST, glutathione *S*-transferase; IU, international units; MDCK, Madin-Darby canine kidney; S9, 9000 × *g* supernatant from rat liver; SCE, sister chromatid exchange; UDS, unscheduled DNA synthesis.

^a No inhibition by indomethacin, suggesting absence of activation by prostaglandin H synthase.

^b Clastogenic effects due to changes in intracellular calcium.

contain an ochratoxin A or ochratoxin A metabolite moiety and that at least some of the adducts might have been due to ochratoxin A-induced cytotoxic effects generating reactive oxygen species (ROS). Other studies *in vitro*, using purified DNA and mononucleotides incubated with kidney or liver microsomes from mouse and rabbit, ochratoxin A and either reduced nicotinamide adenine dinucleotide phosphate (NADPH) or arachidonic acid as cofactors, indicated that oxidative damage to DNA might not be the only source of the presumed adducts (Obrecht-Pflumio & Dirheimer, 2000).

In contrast to these results with ^{32}P -postlabelling methods, others reported that the level of covalent binding of [^3H]ochratoxin A or its metabolites to DNA was below the limit of detection of scintillation counting in rodent kidney and liver *in vivo* or in rat or human hepatocytes *in vitro* (Rasonyi, 1995; Schlatter et al., 1996; Gautier et al., 2001; Gross-Steinmeyer et al., 2002). However, Gautier et al. (2001) did report a small increase in DNA adduct levels in male Fischer 344 rats treated orally with [^3H]ochratoxin A compared with controls, using the ^{32}P -postlabelling assay, and suggested that these may be due to products derived from ochratoxin A-mediated cytotoxicity.

In the more recent studies, using the ^{32}P -postlabelling assay, with nuclease P1-enrichment, thin layer chromatography (TLC) analysis showed that several spots could be detected in chromatograms of the DNA fraction of liver and kidneys of rats and mice chronically exposed to high levels of ochratoxin A (0.4–2.5 mg/kg bw per day) for 2 years. Adduct levels were almost undetectable at 0.6 mg/kg bw, were low at 1.2 mg/kg bw but were quantifiable at 2.5 mg/kg bw following 48 or 72 h of incubation. The largest number of TLC spots was found in chromatograms of DNA from male DA rats, a strain highly susceptible to ochratoxin A-induced kidney carcinogenesis, with fewer spots in females and in Lewis rats. The higher relative adduct level in male mouse kidney as compared with the liver and the female kidneys is consistent with the observation that male mice are more susceptible than females to renal carcinogenesis (Faucet et al., 2004; Manderville, 2005; Pfohl-Leszkowicz & Castegnaro, 2005). As pointed out by EFSA (European Food Safety Authority, 2006), the chemical structure of the spots visible in the TLC chromatograms was not identified, and hence there was no indication that the spots represent genuine ochratoxin A–DNA adducts.

The possible role of lipid peroxidation (LPO) in the formation of DNA adducts has been considered. In studies cited by EFSA (European Food Safety Authority, 2006) involving substances other than ochratoxin A that generate LPO products, several exocyclic DNA adducts derived from LPO products have been identified, such as ethenobases, formed by reaction of *trans*-4-hydroxy-2-nonenal with DNA. Another major DNA lesion associated with LPO is the adduct formation by the reaction of malondialdehyde with guanine. As EFSA has pointed out (European Food Safety Authority, 2006), it therefore cannot be excluded that the DNA adducts shown as spots observed in studies with ochratoxin A using the ^{32}P -postlabelling technique are due to these adducts and hence are not related to ochratoxin A itself, but instead are formed via an indirect mechanism. This hypothesis is supported by the observation that treatment of animals with antioxidants prior to ochratoxin A exposure significantly reduced the number and intensity of DNA fragments formed (Pfohl-Leszkowicz et al., 2002). However, one group has used the ^{32}P -postlabelling assay to search for LPO-related adducts in the kidneys of rats treated with ochratoxin A at doses of 0, 0.25, 0.5, 1 or 2 mg/kg bw for 5 days/week for 2 weeks but could not detect any, nor was there any other evidence of LPO (Mally et al., 2005a).

It has also been postulated that DNA adducts might originate from a reaction between the hydroquinone derived from ochratoxin A and cellular DNA. The presence of hydroquinone has been demonstrated in rat urine (Mally et al., 2004).

To investigate this hypothesis, DNA adduct formation was studied *in vitro* using [^3H]ochratoxin A as substrate and horseradish peroxidase and prostaglandin H synthase as activation system to facilitate the formation of the hydroquinone. Using liquid scintillation counting, no DNA adducts were found (Gautier et al., 2001).

It has, however, been shown that C8 ochratoxin A-3'-monophosphate-deoxyguanosine (OTA-3'-dGMP) adducts can be generated by photoirradiation (Faucet et al., 2004). It was hypothesized that, *in vivo*, an ochratoxin A phenoxy radical, ochratoxin quinone (OTQ), is formed by oxidative dechlorination, and the radical could interact with DNA to form such an adduct. OTQ could be reduced to ochratoxin hydroquinone (OTHQ) and OTHQ autoxidized back to OTQ (Dai et al., 2004). While the chemical structure of ochratoxin A-DNA adducts formed *in vivo* has not been completely characterized, it has been suggested that they represent C-C8 and O-C8 OTA-3'-dGMP, because these synthetic adducts appear to co-chromatograph with adducts found using the ^{32}P -postlabelling technique in kidneys of pig following subacute exposure (0.20 mg/kg bw) and in rats after chronic exposure (3 times weekly, 2 years; total dose 100 mg/kg bw) (Faucet et al., 2004; Pfohl-Leszkowicz & Castegnaro, 2005). However, as EFSA has pointed out (European Food Safety Authority, 2006), the co-chromatography was demonstrated with only one set of chromatographic conditions and thus remains to be confirmed.

Further work by the same group, using the ^{32}P -postlabelling technique, has shown that a quinone/hydroquinone redox couple derived from ochratoxin A may play a role in ochratoxin A-mediated genotoxicity. The adduction properties of ochratoxin A and hydroquinone were compared in salmon sperm DNA, in the absence and presence of pig kidney microsomes as the metabolic activation system. In the absence of metabolic activation, ochratoxin A did not generate DNA adducts, but hydroquinone did generate DNA adducts. The authors ascribed these adducts to covalent DNA adduction by quinone, generated through autoxidation of the hydroquinone precursor, OTHQ. The quinone-mediated adduct spots noted with hydroquinone were also observed when ochratoxin A was incubated with pig kidney microsomes and NADPH, suggesting that ochratoxin A undergoes oxidative activation to quinone by cytochrome P450 or enzymes with peroxidase activity. Adduct formation was also compared in human kidney and bronchial epithelial (WI26) cell lines, in the presence of metabolic activation. DNA adducts were formed in a dose- and time-dependent manner both with ochratoxin A and with hydroquinone, but the rate of adduct formation was faster with hydroquinone, presumably reflecting a more ready formation of quinone from hydroquinone (Tozlovanu et al., 2006). However, quinone-mediated adducts do not co-migrate with C-C8 and O-C8 OTA-3'-dGMP standard adducts, suggesting that the quinone electrophile is not responsible for the formation of C8-deoxyguanosine (C8-dG) adducts that are produced by photoactivation of ochratoxin A. Moreover, DNA adducts deriving from hydroquinone are mainly formed after biotransformation of ochratoxin A by CYP2C9 enzyme (with epoxigenase activity), whereas C8-dG adducts are formed after biotransformation by 5-lipoxygenase, which is a microsomal glutathione (GSH) enzyme (Pfohl-Leszkowicz & Manderville, 2007).

A study on the possible involvement of biotransformation of ochratoxin A via the GSH pathway in production of DNA adducts has been conducted in the opossum kidney (OK) cell line derived from the proximal tubule of American

opossum. The cells were treated with ochratoxin A alone or in the presence of agents such as 2-mercaptoethane sulfonate and *N*-acetyl-L-cysteine, which reduce oxidative stress by increasing free thiols in the kidney, buthione sulfoximine, an inhibitor of GSH synthase, and α -amino-3-chloro-4,5-dihydro-5-isoxazole acetic acid, an inhibitor of gamma glutamyl transpeptidase. None of these agents reduced ochratoxin A-induced cytotoxicity in the OK cells, and all enhanced ochratoxin A biotransformation. Both the ochratoxin A metabolic profiles produced and the nature of the DNA adducts formed during incubation with these agents differed qualitatively and quantitatively according to the agent. The findings suggested that the mechanism by which ochratoxin A results in DNA adduct formation is closely related to its biotransformation by a pathway involving quinone derivatives and GSH conjugation (Faucet-Marquis et al., 2006).

Other experiments have also aimed to identify the chemical structure of potential ochratoxin A adducts. In *in vitro* studies, incubation of ochratoxin A with DNA or dG in the presence of various activation systems did not reveal any adducts, neither by ^{32}P -postlabelling nor by liquid chromatography/tandem mass spectrometry (LC-MS/MS). Subsequently, DNA was isolated from the livers and kidneys of male Fischer rats treated with a single dose of [^{14}C]ochratoxin A (0.5 mg/kg bw) and analysed by ^{14}C -accelerated mass spectrometry. No significant difference between control group and treated group could be observed in ^{14}C activity in isolated DNA from treated animals, and no specific ochratoxin A–DNA adducts were detected with ^{14}C -accelerated mass spectrometry (Mally et al., 2004). The EFSA opinion pointed out that a concern in the interpretation of these results is that DNA adducts may have been repaired, as DNA was isolated 72 h after a single treatment with a relatively low dose, which is in contrast to other studies, in which DNA was isolated within 24 h after exposure to ochratoxin A (European Food Safety Authority, 2006).

In an effort to improve on the previous methodology, the same group has developed a stable isotope dilution LC-MS/MS method to analyse for ochratoxin A–dG adduct (dGuoOTA) in kidney DNA. DNA was isolated from the kidneys of male Fischer 344 rats treated orally with 210 μg ochratoxin A/kg bw per day for 90 days. The dose selected is known to cause renal tumours if given to rats chronically. Despite the sensitivity of the method (limit of detection [LOD] was calculated to be as low as 3.5 dGuoOTA/ 10^9 nucleotides), dGuoOTA was not detected in the kidney DNA of treated animals (Delatour et al., *in press*).

(b) DNA damage and repair

As summarized previously by this Committee, there was no evidence of DNA repair as a result of possible DNA damage in bacteria, whereas DNA single-strand breaks were consistently induced in cultured mammalian cells and were also observed *in vivo* in spleen, liver and kidney cells of mice after intraperitoneal injection of ochratoxin A. DNA repair, manifested as unscheduled DNA synthesis, was observed in most studies with primary cultures of rat and mouse hepatocytes, porcine epithelial cells from bladder and human urothelial cells (Annex 1, reference 153).

In the more recent studies, there is further evidence of DNA damage, as assessed in the comet assay both in vitro, in mouse fibroblasts, rat liver, dog kidney, human kidney and human urothelial cells (Ehrlich et al., 2002; Lebrun & Föllmann, 2002; Lebrun et al., 2006; Simaro Doorten et al., 2006; Arbillaga et al., 2007a, 2007b), and in vivo, in rat liver, kidney and spleen (Mally et al., 2005b; Zeljezic et al., 2006). In some of these studies, the amount of DNA damage was enhanced by agents that highlight oxidative damage.

(c) *Gene mutation*

As summarized previously by this Committee, most tests for induction of gene mutations in bacteria showed no effect of exposure to ochratoxin A, whereas two studies showed positive results (Annex 1, reference 153). Two recent studies on gene mutation in bacteria were negative (Ehrlich et al., 2002; Föllmann & Lucas, 2003). The Committee noted that in none of the bacterial studies was ochratoxin A tested up to cytotoxic concentrations or alternatively up to the highest concentration (5 mg/plate) recommended in current test guidelines. The Committee also noted that in those studies utilizing *Salmonella* strains TA102, TA104 or TA2638 or *Escherichia coli* strain WP2, all of which are known to be sensitive to oxidative stress, the results were negative.

In mammalian cells, gene mutations were not induced in three studies, whereas positive results were observed in one (Annex 1, reference 153; Föllmann & Lucas, 2003).

(d) *Chromosomal aberration*

As summarized previously by this Committee, sister chromatid exchange was induced in two of four studies in vitro but not in a single study in vivo after gavage of a range of doses that included cytotoxic doses (Annex 1, reference 153). A more recent in vitro study was positive (Lioi et al., 2004).

As summarized previously by this Committee, in tests for chromosomal aberrations in vitro, there was one positive and one negative study; in tests for micronucleus formation in vitro, there were two positive results. Two more recent in vitro tests on chromosomal aberration (Lioi et al., 2004) and micronucleus formation (Ehrlich et al., 2002) were also positive. In vivo, as summarized previously by this Committee, chromosomal aberrations were induced in mice (Annex 1, reference 153). In a more recent study, results in rats were negative (Mally et al., 2005b).

2.2.4 *Reproductive and developmental toxicity*

No adequate studies on the reproductive toxicity of ochratoxin A were available for review. Several studies on developmental toxicity, showing that ochratoxin A can cross the placenta and that it is embryotoxic and teratogenic in rats and mice, were described previously by this Committee (Annex 1, reference 153). Studies published since 2001 are summarized below.

(a) *Rats*

Groups of 10 Wistar rats were given ochratoxin A by oral gavage at doses of 0, 0.125, 0.25, 0.50 or 0.75 mg/kg bw per day on days 6–15 of gestation. Ochratoxin A caused increases in embryonic and fetal death at 0.25 mg/kg bw per day and above, as seen by increased incidences of dams with complete resorption/abortion and dams with some resorptions and a reduced number of live fetuses. These effects were dose related and reached statistical significance at the highest dose. Fetal weight and crown–rump length were also reduced in a dose-dependent way, and the reductions were statistically significant at the highest dose. Gross malformations and skeletal and visceral anomalies were all increased at all doses, in a dose-dependent manner, and the increases were statistically significant at 0.5 and 0.75 mg/kg bw per day. Gross malformations included exencephaly, incomplete closure of skull, micrognathia, micromelia, curly tail, scoliosis and small hind portion. Skeletal anomalies included incomplete ossification in a number of bones and fused, wavy or branched ribs. Visceral defects included hydrocephalus, microphthalmia, dilated renal pelvis, hydronephrosis and cryptorchid testis. Histological examination of liver, kidney, brain and eyes in subsets of fetuses undergoing visceral examination showed an increased incidence of lesions in those from dams given 0.25 mg/kg bw per day and above. The lesions included oedema, fibrosis and epithelial degeneration in the kidney, hepatocyte degeneration, bile duct proliferation, hypoplasia of the cerebellum and defects of the lens and retina (Wangikar et al., 2004a, 2004b). The same group has shown that the minimum teratogenic dose of ochratoxin A in Wistar rats, when given as a single oral gavage dose on one of days 6–15 of gestation, is 2.75 mg/kg bw per day. The most sensitive days for inducing teratogenicity were day 6 and day 7 of gestation (day of finding sperm in vaginal smear designated day 0) (Patil et al., 2006).

(b) *Rabbits*

Groups of five New Zealand White rabbits were given ochratoxin A by oral gavage at doses of 0, 0.025, 0.05 or 0.10 mg/kg bw per day on days 6–18 of gestation. In the highest dose group, there were significant decreases in fetal weight and number of live fetuses. There was an increased incidence of fetuses with malformations or anomalies (knuckling of fetlock, rudimentary tail or agenesis of tail, wavy ribs, hydrocephalus, microphthalmia, agenesis of kidney, reduced ossification of the skull and failed ossification in the caudal vertebrae). Histological examination of liver, kidney, brain and eyes showed a dose-related increase in lesions in fetal liver and kidney. None of the increases in malformations, anomalies or pathological lesions reached statistical significance on a per litter basis, owing to the small number of litters per dose group (Wangikar et al., 2005).

2.2.5 *Special studies*

(a) *Neurotoxicity*

Ochratoxin A is neurotoxic *in vitro* and *in vivo* in rats given ochratoxin A orally for 7–35 days at doses of 120 and 290 µg/kg bw per day, as described previously by this Committee (Annex 1, reference 153). More recent studies have been

published, most of which have been described by EFSA (European Food Safety Authority, 2006), and are briefly summarized below.

(i) *In vitro*

In cultured rat aggregated brain cells, 10–20 nmol ochratoxin A/l was shown to increase the expression of genes involved in the brain inflammatory system (messenger ribonucleic acid [mRNA] of peroxisome proliferator-activated receptor, haem oxygenase-1 and inducible nitric oxide synthase) and to reduce the expression of glial fibrillary acidic protein, a constituent of the intermediate filaments in astrocytes (Zurich et al., 2005).

In cultured rat embryonic midbrain cells, 0.5 and 1 µg ochratoxin A/ml caused a reduction in the number of viable cells, an induction of transcription factors activator protein-1 and nuclear factor-kappa B (NF-κB) activation as well as an inhibition of neurite outgrowth at the higher concentration (Hong et al., 2002).

In cultured proliferating neural stem/progenitor cells from adult mouse hippocampus, ochratoxin A added at concentrations of 0.01–100 µg/ml caused dose- and time-dependent (6–72 h) decreases in viability of both proliferating and differentiating cells. Proliferating cells were more vulnerable to ochratoxin A toxicity despite robust DNA repair and anti-oxidative responses. Preconditioning of the cells with the pro-oxidant diethyl maleate increased DNA repair activity and provided a moderate degree of neuroprotection. The authors speculated that these responses may contribute to impaired hippocampal neurogenesis in vivo (Sava et al., 2007).

(ii) *In vivo*

In mice, striatal dopamine was depleted in a dose-dependent manner following single intraperitoneal doses of 3–6 mg ochratoxin A/kg bw. Oxidative stress, oxidative DNA damage and a transient inhibition of oxidative DNA repair were also seen in the cerebellum, cortex, hippocampus, midbrain, caudate/putamen and pons/medulla (Sava et al., 2006).

Groups of 10 young (aged 12 weeks) and old (aged 27–20 months) female SPF Wag rats were given ochratoxin A by oral gavage at doses of 0, 70, 340 or 1680 µg/kg bw per day for 4 weeks. A significant increase in mortality occurred at the highest dose in both young and old rats. Vacuolation of the white brain matter (cerebellar medulla and ventral parts of the brain stem) was increased at all dose levels, reaching statistical significance in young rats at 340 and 1680 µg/kg bw per day and in old rats at 70 and 340 µg/kg bw per day (Dortant et al., 2001).

Groups of eight rats received 0 or 289 µg ochratoxin A/kg bw per day or the same dose of ochratoxin A plus melatonin (10 mg/kg bw per day) orally via the drinking-water for 4 weeks. Significant reductions in the concentrations of hippocampal *N*-methyl-D-aspartate receptor subunits 2A and 2B (involved in memory and learning processes) were reported in ochratoxin A-treated rats compared with controls. Melatonin partially protected against the reduction in *N*-methyl-D-aspartate receptor subunits caused by ochratoxin A (Delibas et al., 2003).

(b) Immunotoxicity

Ochratoxin A is immunotoxic *in vitro* and *in vivo*, as described previously by this Committee (Annex 1, reference 153). More recent studies have been described by EFSA (European Food Safety Authority, 2006) and are briefly summarized below.

(i) *In vitro*

In rat lymphoid cells exposed to concentrations of ochratoxin A of 0, 0.5, 2 or 20 $\mu\text{mol/l}$ (equivalent to 0, 0.2, 0.8 or 8 mg/l), the cytotoxic activity of natural killer cells was dose-dependently decreased, reaching statistical significance at the highest dose. The cytotoxic T lymphocyte activity was significantly decreased at the lowest concentration only. The bacteriolytic activity of macrophages varied only slightly, and the proliferative response of lymphocytes to concanavalin A and lipopolysaccharide was not affected (Alvarez-Erviti et al., 2005).

In a study using very high concentrations, ochratoxin A inhibited the mitogen (concanavalin A)-induced lymphocyte proliferation in purified lymphocytes from piglets. The IC_{50} was 1.3 $\mu\text{mol/l}$ (equivalent to 0.52 mg/l), which is several orders of magnitude higher than the highest plasma levels found in humans (Keblys et al., 2004).

(ii) *In vivo*

Groups of 10 young (aged 12 weeks) and old (aged 27–20 months) female SPF Wag rats were given ochratoxin A by oral gavage at doses of 0, 70, 340 or 1680 $\mu\text{g/kg bw per day}$ for 4 weeks. A significant increase in mortality occurred at the highest dose in both young and old rats. No old animals were available for testing of immune parameters at the highest dose because of mortality. Decreased immunoglobulin G (IgG) levels were seen at 340 $\mu\text{g/kg bw per day}$ in both age groups and also at 1680 $\mu\text{g/kg bw per day}$ in the young rats. Ochratoxin A also induced a dose-related reduction in the splenic T cell fraction of young rats, which was statistically significant at the highest dose only (Dortant et al., 2001).

Male Wistar rats were given ochratoxin A by oral gavage at doses of 0, 50, 150 or 450 $\mu\text{g/kg bw per day}$ for 28 days, and several immune function assays were performed at the end of treatment. The study was conducted according to Organisation for Economic Co-operation and Development (OECD) Test Guideline 407 (1995). The natural killer cell activity was strongly affected; the per cent lysis of the target cells was significantly reduced in all treated groups, and natural killer cell activity was completely suppressed at the highest dose. The response of splenocytes to sheep red blood cells was decreased in an apparently dose-dependent manner, but none of the reductions in response were statistically significant. The cytotoxic T lymphocyte activity was lowered in the 50 $\mu\text{g/kg bw per day}$ group only. The bacteriocidal activity of macrophages was significantly reduced at 50 and 450 $\mu\text{g/kg bw per day}$, but not at the intermediate dose of 150 $\mu\text{g/kg bw per day}$. Histopathology of the thymus and spleen did not show significant differences from control (Alvarez et al., 2004).

(c) *Mechanisms of toxicity in the kidney*

Induction of collagen secretion (a marker of fibrosis) has been shown in the OK proximal tubular cell line and in cultured primary human renal proximal tubular cells exposed to ochratoxin A. Collagen secretion was both time and dose dependent, as was the induction of cell toxicity (Sauvant et al., 2005a).

Exposure of a rat kidney proximal tubular cell line (NRK-52E) to ochratoxin A at concentrations of 100 and 1000 nmol/l resulted in changes typical of chronic interstitial nephropathy, including loss of epithelial tightness, reduced cell number due to necrosis, as measured by release of lactate dehydrogenase (LDH) or DNA ladder formation, and apoptosis, as measured by caspase-3 activation. Ochratoxin A induced NF- κ B activity (a marker of inflammatory activity), collagen secretion and generation of alpha smooth muscle actin (a marker of epithelial mesenchymal transition). It also induced three mitogen-activated protein kinases (MAPK): extracellular signal-regulated kinase 1 and 2 (ERK 1/2) (thought to be promitotic), c-jun N-terminal kinase (JNK) and extracellular-regulated protein kinase 38 (p38) (thought to be involved in apoptosis, fibrosis and inflammation). All three were induced in a dose-dependent manner (Sauvant et al., 2005b).

Exposure of OK and NRK-25E cells to ochratoxin A in the presence of an inhibitor of ERK 1/2 showed that the toxicity of ochratoxin A, as measured by cell number, protein, epithelial tightness, apoptosis and necrosis, was increased. Biomarkers of inflammation, fibrosis and epithelial mesenchymal transition were also enhanced in the presence of the inhibitor of ERK 1/2, compared with ochratoxin A alone. The authors speculated that the toxicity of ochratoxin A may be influenced by the balance between the profibrotic ERK 1/2 and the antifibrotic JNK and p38, which are not affected by inhibition of ERK 1/2, and that naturally occurring inhibitors of ERK 1/2, such as anthocyanidins, may amplify the effects of ochratoxin A (Sauvant et al., 2005c).

The effects of ochratoxin A on human kidney proximal tubule cells and on human lung fibroblasts in primary culture have been studied by the same group. The cells were exposed to ochratoxin A at concentrations of 0.3 nmol/l up to 10 μ mol/l for 2, 5 or 14 days. The activities of caspase-3 and LDH were measured as indicators of apoptosis and necrotic cell death. Cellular protein content, collagen, fibronectin secretion and the activation of transcription factor NF- κ B were also measured. Qualitatively similar effects were seen in the two types of cells tested, but kidney cells were around 10 times more sensitive than fibroblasts with respect to increases in caspase-3 and LDH release. Very low concentrations (0.3–10 nmol/l) of ochratoxin A led to hypertrophy in kidney cells exposed for 14 days. A fibrotic reaction was confirmed by increases in NF- κ B activity, collagen III and fibronectin secretion in the kidney cells, which were absent in the fibroblasts. The authors commented that the changes seen in cultured kidney cells exposed for 14 days at low nanomole per litre or sub-nanomole per litre concentrations may indicate a need for reassessment of the effects of ochratoxin A consumption in humans, which generally leads to average blood concentrations around 1 nmol/l (Schwerdt et al., 2007).

(d) *Epigenetic modes of action*

(i) *Oxidative DNA damage*

Earlier work on oxidative stress, as a potential mechanism of ochratoxin A toxicity that could lead indirectly to damage to DNA and eventually tumorigenesis, has been summarized previously by this Committee (Annex 1, reference 153). Commenting on the discrepant data on mode of action in 2001, the Committee noted that the oxidative stress hypothesis provided an alternative explanation to the hypothesis of direct damage to DNA and was more consistent with the observations. More recent data on oxidative stress are summarized below.

In vitro

Ochratoxin A-induced (oxidative) DNA damage, cytotoxicity (necrosis, growth inhibition, apoptosis) and modulation of GSH were investigated in cell lines (V79, CV-1) and in primary rat kidney (PRK) cells. After 24 h incubation, viability of V79 but not CV-1 cells was strongly decreased by ochratoxin A at concentrations above 2.5 $\mu\text{mol/l}$. Strong inhibition of growth was observed in both cell lines, with an IC_{50} around 2 $\mu\text{mol/l}$. Apoptosis was increased in CV-1 cells (V79 not tested) at concentrations above 1 $\mu\text{mol/l}$. Specific oxidative DNA damage (the difference between total DNA damage and basic DNA damage) was assessed by the comet assay after additional treatment with repair enzyme formamido-pyrimidine-DNA-glycosylase (Fpg) or endonuclease III, which recognize oxidized purine and pyrimidine bases, cut them out and nick the DNA in the respective sites, resulting in additional DNA damage. Specific oxidative DNA damage was induced in all cell systems by ochratoxin A at low concentrations, from 0.25 $\mu\text{mol/l}$ upwards in V79 cells and from 1 $\mu\text{mol/l}$ upwards in CV-1 and PRK cells. Basic DNA damage was not increased or was seen only at longer incubations or higher concentrations of ochratoxin A. Total GSH levels in CV-1 cells were significantly reduced after 1 h of incubation at 100 $\mu\text{mol/l}$, but slightly increased after 24 h. The authors concluded that the observations of oxidative DNA damage and cytotoxicity at low concentrations of ochratoxin A suggest that oxidative stress and a reactive proliferative response play a role in its renal carcinogenicity (Kamp et al., 2005a).

In a cultured human renal proximal tubular cell line, HK-2, a concentration-related increase in the level of ROS compared with controls was observed after 6 h exposure to ochratoxin A at 50–600 $\mu\text{mol/l}$. Pretreatment with *N*-acetyl-L-cysteine, an antioxidant ROS scavenger, decreased the level of ROS and increased cell survival. *N*-Acetyl-L-cysteine also reduced DNA damage at 50–200 $\mu\text{mol/l}$, as measured by the comet assay, but not at higher ochratoxin A concentrations of ≥ 400 $\mu\text{mol/l}$, at which the generation of ROS outpaced the reduction by *N*-acetyl-L-cysteine (Arbillaga et al., 2007a).

In a follow-up study, gene expression was assessed in HK-2 cells exposed to ochratoxin A at 50 $\mu\text{mol/l}$ for 6 or 24 h. After 6 h, when slight cytotoxicity was evident (83% survival), genes involved in the mitochondrial electron transport chain were up-regulated. After 24 h, when there was greater cytotoxicity (51% survival), genes implicated in the oxidative stress response were also up-regulated. Increases

in the level of ROS and oxidative DNA damage as assessed by the comet assay were also evident at both time intervals and were more pronounced in the presence of higher cytotoxicity. There was no effect at either time interval on genes implicated in DNA damage response (repair, apoptosis and cell cycle control) (Arbillaga et al., 2007b).

Pig kidney epithelial (PK15) cells were exposed to ochratoxin A at concentrations of 0, 0.05, 0.5 and 5 µg/ml for 24 or 48 h. At the highest concentration, cell viability was reduced by 35% after 24 h and LPO increased by 56% and 85% at 24 and 48 h, respectively, compared with controls. GSH was significantly reduced by 18% after 24 h of exposure to 0.05 µg/ml (Klaric et al., 2007).

The effects of pretreatment with antioxidant catechins, epigallocatechin gallate and epicatechin gallate, on ochratoxin A toxicity were investigated in a pig kidney cell line (LLC-PK1) that has been used as a model for kidney toxicity. Pretreatment with catechins at 1–60 µmol/l was combined with subsequent exposure to 10–20 µmol ochratoxin A/l, and cell viability, ROS production and DNA fragmentation were measured. The pretreatment with catechins prevented ochratoxin A-induced cell death and reduced ochratoxin A-induced ROS production and DNA fragmentation. In tests of the antioxidant activity of the catechins alone, epigallocatechin gallate and epicatechin gallate showed good free radical scavenging power, in accord with their inhibition of ROS production (Costa et al., 2007).

In vivo

Ochratoxin A was shown to induce DNA strand breaks as assessed by comet assay in liver, kidney and spleen of Fischer 344 rats given doses of 0, 0.25, 0.50, 1.0 or 2.0 mg/kg bw per day for 2 weeks, 5 days/week. In liver and kidney, the extent of DNA damage was further enhanced in a dose-dependent manner in the presence of the repair enzyme Fpg, which converts oxidative DNA damage into strand breaks, suggesting the presence of oxidative DNA damage (Mally et al., 2005b).

The occurrence of oxidative DNA damage was investigated *in vivo* in male Fischer 344 rats dosed orally with ochratoxin A at 0, 0.03, 0.1 or 0.3 mg/kg bw per day for 4 weeks. The doses used were within the range known to have caused kidney tumours in rats in a 2-year study. Oxidative DNA damage was assessed by the comet assay, with and without use of the repair enzyme Fpg. There was no effect on basic DNA damage, but oxidative DNA damage was detected with Fpg treatment in kidney and liver at all doses tested (Kamp et al., 2005b).

Male Fischer 344 rats were fed ochratoxin A in the diet for up to 2 years at concentrations giving an initial intake at the initial body weight of 175 g of 300 µg/kg bw per day, declining and then held at 100 µg/kg bw per day when the animals reached a body weight of 333 g. Renal tumours appeared during the last 6 months of the study in 25% of the animals. The gene expression profile in the liver and kidney was studied in groups of five animals at intervals of 7 and 21 days and 4, 7 and 12 months after the commencement of treatment. In both organs, down-regulation of genes was the predominant effect. Two main clusters of genes could be identified that were continuously up-regulated or down-regulated over the five

time points studied. The kidney showed more modulation than the liver, possibly reflecting the elevated content and prolonged residence time of ochratoxin A in that organ. In the kidney, many genes expected to be induced by oxidative stress were significantly down-regulated, whereas no such modulation was seen in the liver; many of these genes share the antioxidant regulatory element in their promoter region, which is under transcriptional control by nuclear factor-erythroid 2 p45-related factor (Nrf2). Genes involved in fatty acid metabolism and cytochrome P450 were also selectively down-regulated in the kidney; many of these genes share the common promoter hepatocyte nuclear factor 4- α (HNF4 α), and several genes from other functional classes also under transcriptional control by HNF4 α were also down-regulated, as was the mRNA specific for HNF4 α . Several genes known as markers of kidney injury and cell regeneration were up-regulated. Only small changes were seen in the expression of genes involved in DNA synthesis and repair or genes induced as a result of DNA damage. Very little change was seen in genes associated with apoptosis. There was a strong down-regulation of regucalcin, which is known to play a role in the regulation of intracellular calcium homeostasis in kidney tubule cells. The authors concluded that several epigenetic mechanisms may be involved in ochratoxin A carcinogenicity, including alteration of calcium homeostasis and disruption of pathways regulated by transcription factors HNF4 α and Nrf2. In particular, depletion of Nrf2-regulated enzymes is likely to impair cellular defences against oxidative stress, but the authors acknowledge that there is no direct evidence of a link between the gene expression changes observed and kidney tumour formation (Marin-Kuan et al., 2006).

In a follow-up study on the same ochratoxin A-treated animals, it was confirmed that the loss of expression of Nrf2-regulated genes seen in the previous study (Marin-Kuan et al., 2006) was accompanied by decreased protein expression of several markers of the Nrf2-regulated gene battery in the kidney. Inhibition of Nrf2 activity was also shown *in vitro* by exposure of rat liver (primary hepatocytes and RL-34) cells and kidney (NRK) cells to ochratoxin A at concentrations of 1.5–6 $\mu\text{mol/l}$. Down-regulation of Nrf2 gene expression was accompanied by oxidative DNA damage both *in vitro* and *in vivo*, as measured by the formation of abasic sites in DNA. *In vitro*, the effects of ochratoxin A were prevented by pretreatment of the cell cultures with inducers of Nrf2 activity. The authors concluded that reduction of cellular defences against oxidative stress via Nrf2 inhibition may be a plausible mechanism for ochratoxin A nephrotoxicity and carcinogenicity (Cavin et al., 2007).

Studies on the protective effect of substances with antioxidant and free radical scavenging activities have provided further evidence of the possible role of oxidative stress in ochratoxin A-induced toxicity.

Pretreatment or co-treatment of rats with melatonin at doses of 10–20 mg/kg bw per day has a preventive effect on ochratoxin A-induced liver and kidney toxicity (Aydin et al., 2003) and on indicators of oxidative stress, such as changes in liver and kidney GSH peroxidase, superoxide dismutase and LPO, as measured by production of malondialdehyde after 4 weeks of ochratoxin A treatment (Meki & Hussein, 2001; Ozcelik et al., 2004; Abdel-Wahhab et al., 2005; Sutken et al., 2007).

Red wine has been shown to exert a protective effect against ochratoxin A nephrotoxicity in rats by limiting oxidative damage as measured by renal lipohydroperoxides, reduced and oxidized GSH and renal superoxide dismutase activity. The protective components in red wine may be the antioxidant flavonoids (Bertelli et al., 2005). The same group has shown that red wine reduces ochratoxin A-induced renal cortical fibrosis in rats during its early developmental stage by inhibiting the molecular mechanisms of the tubular cell transition from epithelial phenotype to myofibroblasts, which are the major effector cells responsible for deposition of interstitial extracellular matrix in pathological fibrosis. However, red wine has no effect on the mechanisms that predominate later to induce fibrosis under chronic ochratoxin A treatment (Gagliano et al., 2005). Conversely, others have shown, using an *in vitro* model, intestinal Caco-2/TC7 cells, that dealcoholated red wine at a 1:10 dilution in the medium may act synergistically with ochratoxin A (at a concentration of 100 $\mu\text{mol/l}$) to trigger an apoptotic cascade in such cells (Ranaldi et al., 2007).

The potential protective effect of 2-mercaptoethane sulfonate, which reduces oxidative stress by increasing free thiols in the kidney, on renal toxicity and carcinogenicity induced by ochratoxin A has been examined in a long-term rat study. 2-Mercaptoethane sulfonate significantly reduced karyomegaly but did not reduce the incidence of renal tumours (Pfohl-Leschkowicz & Manderville, 2007).

(ii) Effects on cell–cell signalling and cell division

In vitro

Since disruption of cell–cell signalling has been implicated in contributing to the mechanism of carcinogenicity of a number of nephrotoxic agents, the effects of ochratoxin A on gap junction intercellular communication (GJIC) and cell adhesion have been investigated. Such changes may, however, be the consequence rather than the cause of the toxicity of ochratoxin A. Madin Darby canine kidney (MDCK) cells were exposed for up to 24 h in culture to ochratoxin A at concentrations from 1 to 50 $\mu\text{mol/l}$. After 4 h exposure, when there was no evidence of cytotoxicity, GJIC was significantly affected, as measured by the reduction in distance of dye transfer, at 10 $\mu\text{mol/l}$ and above. After 24 h exposure to 25 or 50 μmol ochratoxin A/l, there was almost complete inhibition of GJIC compared with 4 h, but this was accompanied by substantial cytotoxicity, suggesting disruption of monolayer integrity. However, inhibition of GJIC was also observed at lower, non-cytotoxic concentrations (1 and 10 $\mu\text{mol/l}$) after 24 h. Gap junctions, which allow exchange of signalling molecules between cells, are formed by oligomerization of connexin (Cx) proteins. Inhibition of GJIC by ochratoxin A was associated with a small, but dose-dependent, decrease in Cx43 protein levels at all time points, probably via down-regulation of Cx43 expression, rather than by changes in total cellular phosphorylation. Intracellular amounts of the adherens junction proteins, E-cadherin and β -catenin, were not affected by ochratoxin A. Changes were seen in localization of these two adherens at 25 and 50 $\mu\text{mol/l}$, in association with cytotoxicity, but it was not possible to determine whether these changes may have

preceded cell death. The authors concluded that disruption of cell–cell signalling may contribute to the toxicity and carcinogenicity of ochratoxin A (Mally et al., 2006).

Immortalized human kidney epithelial cells, a model in which the morphological changes closely resemble those in the rat kidney *in vivo*, were exposed to ochratoxin A at concentrations of 0, 1, 10 and 50 $\mu\text{mol/l}$. Significant time- and dose-dependent changes were observed at concentrations of 10 and 50 $\mu\text{mol/l}$. These concentrations are in the same range as plasma concentrations after repeated administration of ochratoxin A to rats at doses known to induce tumours. The changes included increases in apoptosis and decreases in the rate of mitosis. Nuclear morphology showed that the later stages of mitosis (anaphase/telophase) were rarely present, and the number of aberrant nuclei with misaligned chromosomes was significantly increased at 10 and 50 $\mu\text{mol/l}$. There was also formation of giant cells with abnormally large or multiple nuclei, sometimes still connected by chromatin bridges, which is indicative of defects in chromosome segregation and/or cytokinesis. Irregular, asymmetric and sometimes multipolar mitotic spindles were also seen. Disruption of cell–cell and cell–matrix contacts was also observed, associated with decreased phosphorylation of focal adhesion kinase, a central component of integrin-mediated cell adhesion and survival signalling. At the same time, increased activation of the stress-activated MAPK, JNK and the antiapoptotic transcription factor NF- κB was observed. At 50 $\mu\text{mol/l}$, increased phosphorylation of ERK 1/2 was also observed. To further investigate whether the abnormal mitotic spindles were due to ochratoxin A interference with microtubule polymerization, an *in vitro* tubulin polymerization assay was conducted in a cell-free environment. Ochratoxin A was found to inhibit microtubule assembly at 200 and 400 $\mu\text{mol/l}$ but not at 50 or 100 $\mu\text{mol/l}$. The authors hypothesized that the mechanism by which ochratoxin A promotes tumour formation involves interference with microtubule dynamics and mitotic spindle formation, resulting in apoptosis or premature exit from mitosis. This causes blocked or asymmetric cell division, which may favour the occurrence of cytogenetic abnormalities, ultimately leading to tumour formation (Rached et al., 2006).

In vivo

Kidney samples were taken from male Fischer 344 rats fed ochratoxin A in the diet at a dose of 300 $\mu\text{g/kg}$ bw per day for 7 days, 21 days or 12 months and analysed for various cell signalling proteins known to be potentially involved in kidney carcinogenicity. This dose was known to have caused kidney tumours if fed over 2 years (Mantle et al., 2005). Ochratoxin A was found to increase the phosphorylation of atypical protein kinase C (PKC) at all time points compared with controls, the increases being statistically significant at 21 days and 12 months. This was correlated with a selective downstream activation of the MAPK extra-cellular signal–regulated kinases isoforms 1 and 2 (ERK 1/2) and of their nuclear substrate ETS-domain protein 1 (ELK 1/2) and cytosolic substrate, ribosomal-S6 kinase (p90RSK). Analysis of effectors acting upstream of PKC indicated a possible mobilization of the insulin-like growth factor-1 receptor (IGF-1r) and phosphoinositide-dependent kinase-1 system (PDK1). Increased histone deacetylase (HDAC) enzymatic activity associated with enhanced HDAC3 protein

expression was also observed. The authors noted several associations between the changes observed in this rat study and events that have been linked with human renal cancer in other studies: i.e. IGF-1 and subsequent MAPK downstream responses, activation of the MAPK-ERK cascade, increases in IGF-1r and increased atypical PKC activity leading to MAPK activation, which is thought to be a key element in the development of kidney cell carcinoma consequent to inactivation of the tumour suppressor gene von Hippel-Lindau gene product. IGF-1r is also thought to be associated with tumour development in rodents, whereas activation of PKC activity and selective mobilization of ERK 1/2 have been linked to renal cancer in rats. The authors concluded that these results suggest that chronic dietary administration of ochratoxin A to male rats may result in an MAPK response compatible with a selective stimulation of proliferation and stress response, rather than induction of apoptosis, as suggested by earlier in vitro studies (Marin-Kuan et al., 2007).

Wild-type rats and Eker rats heterozygous for a dominant germline mutation in the tuberous sclerosis 2 (*Tsc2*) tumour suppressor gene were exposed orally to ochratoxin A by gavage at a dose of 210 µg/kg bw per day for 1, 3, 7 or 14 days. Renal cell histopathology, cell proliferation and gene expression profiles were investigated in the renal cortex/outer medulla. Ochratoxin A caused slight cortical pathology (preneoplastic lesions) and significantly increased cell proliferation in both strains of rat. A number of genes in the phosphatidylinositol 3-kinase-AKT-*Tsc2*-mammalian target of rapamycin signalling were down-regulated. Eker rats were more sensitive than wild-type rats for all effects. The overall pattern of effects suggested that *Tsc2* may be involved in ochratoxin A toxicity (Stemmer et al., 2007).

2.3 Observations in humans

2.3.1 Biomarkers of exposure

(a) Blood

As the Committee has previously commented, ochratoxin A has a half-life of about 35 days in humans, and the blood concentrations are considered to represent a convenient biomarker of exposure during previous weeks. This biomarker has been used extensively in epidemiological studies. Similar estimates of exposure have been derived from dietary surveys and from blood analyses, suggesting that the latter is a reliable biomarker (Annex 1, reference 153).

In the Committee's previous evaluation (Annex 1, reference 153), the concentrations of ochratoxin A in blood samples from healthy persons ($n = 3717$), obtained in surveys conducted in 17, mainly European, countries, ranged between 0.1 and 40 ng/ml (with an exceptional maximum of 160 ng/ml). The results of more recent surveys, summarized in Table 5, suggest a possible decline in extreme peak values for blood concentrations compared with earlier surveys.

Table 5. Recent studies on the occurrence of ochratoxin A in blood samples of healthy humans

Country	Collecting period	Number of positive samples (%)	Detection limit (ng/ml)	Mean plasma concentration (ng/ml)	Reference
Italy	1995–1996	134/138 (97)	0.1	0.56	Palli et al. (1999)
Croatia	1997–1998	468/983 (48)	0.2	0.30	Peraica et al. (2001)
Norway, Sweden	1997–1998	393/393 (100)	0.01	0.18–0.21	Thuvander et al. (2001)
Morocco	2000	185/309 (60)	0.1	0.29	Filali et al. (2002)
Lebanon	2001–2002	82/250 (33)	0.1	0.17	Assaf et al. (2004)
Poland	1998–1999	30/30 (100)	0.02	1.14	Postupolski et al. (2006)
Chile	2004	62/88 (70)	0.1	0.42–0.88	Munoz et al. (2006)
Argentina (two cities)	2004 2005	125/199 (63) 151/236 (64)	0.012 0.43	0.15	Pacin et al. (2007)

A recent review has considered how exposure to ochratoxin A might relate to the occurrence of Balkan endemic nephropathy, by comparing blood levels in rural endemic versus non-endemic regions of Balkan countries, while acknowledging that systematic data are not available. The authors note that although some studies have indicated that contamination of food by ochratoxin A may be more widespread in rural endemic areas than in non-endemic areas, this is not reflected by markedly elevated blood concentrations of ochratoxin A. Moreover, blood concentrations of ochratoxin A in the same range as those observed in endemic areas have been found in countries with no history of endemic nephropathy. These authors also noted that human blood concentrations of ochratoxin A, even in areas with relatively high dietary exposure, are (at least 2) orders of magnitude below the mean concentration of ochratoxin A in the blood of rats that is known to cause nephrotoxicity and kidney tumours with long-term treatment (Mally et al., 2007).

(b) *Urine*

Recent advances in analytical methods have enabled monitoring of the generally low levels of ochratoxin A in urine.

A total diet study has been conducted in the United Kingdom on 50 individuals, in which composite duplicate-diet samples and plasma and urine samples were analysed over a period of 30 days. The affinity column methodology used achieved sensitivities of 0.001 ng/g in composite foods, 0.1 ng/ml in plasma and 0.01 ng/ml in urine. A significant correlation was found between ochratoxin A intakes and concentrations in urine. Urine was found to be a better indicator of ochratoxin A intakes than plasma, confirming urine as a suitable biomarker to

estimate exposure to ochratoxin A. Urine concentrations in 46/50 samples ranged from <0.01 to 0.058 ng/ml (Gilbert et al., 2001).

In Italy, ochratoxin A was detected in 22 out of 38 urine samples from healthy people in a range of 0.012–0.046 ng/ml (Pascale & Visconti, 2001).

The ochratoxin A content of urine samples from 88 healthy humans living at five settlements in three counties in Hungary was determined. Ochratoxin A was detected in 61% of the samples at an average concentration of 0.013 ng/ml (range: 0.006–0.065 ng/ml) (Fazekas et al., 2005).

Ochratoxin A was detected in 42 out of 60 urine samples taken from healthy persons in Coimbra, Portugal. The concentrations ranged from 0.021 to 0.105 ng/ml, with an average of 0.038 ng/ml (limit of quantification 0.02 ng/ml) (Pena et al., 2006).

(c) *Milk*

Ochratoxin A has been found in human milk at a wide range of concentrations (see Table 6).

Table 6. Studies on the occurrence of ochratoxin A in human milk samples

Country	Number of positive samples (%)	Range of concentration in milk ^a (ng/ml)	Reference
Germany	4/36 (11%)	0.017–0.030	Gareis et al. (1988)
Italy	9/50 (18%)	1.2–6.6	Micco et al. (1991)
Sweden	23/40 (57%)	0.01–0.04	Breitholtz-Emanuelsson et al. (1993)
Sweden	39/39 (100%)	0.09 (average)	Breitholtz-Emanuelsson et al. (1993)
Hungary	38/92 (41%)	0.02–7.3	Kovacs et al. (1995)
Sierra Leone	35/40 (87%)	0.2–337	Jonsyn et al. (1995)
Australia	2/100 (2%)	3–3.6	Apostolou et al. (1998)
Norway	17/80 (21%)	10–182	Skaug et al. (2001)
Italy	198/231 (87%)	6.01 (average)	Turconi et al. (2004)
Brazil	2/50 (4%)	0.01 and 0.2	Navas et al. (2005)
Poland	5/13 (38%)	0.005–0.17	Postupolski et al. (2006)
Egypt	36/50 (72%)	1.89 (average)	Hassan et al. (2006)

^a Unless stated as an average.

Recent evidence suggests that excretion of ochratoxin A into human milk is mediated by breast cancer resistance protein (BCRP). BCRP is a member of the ATP-dependent efflux transporter family, which is highly expressed during lactation in various species, including humans, and is known to be responsible for the excretion of various drugs and xenobiotics into milk (Jonker et al., 2005; Schrickx

et al., 2005; Van Herwaarden et al., 2006). This excretion serves to reduce maternal body burdens but means that breastfed infants are exposed to ochratoxin A.

2.3.2 Biomarkers of effect

Raised urinary β 2-microglobulin levels have been reported in association with impairment of renal tubular function, correlating with high levels of ochratoxin A exposure in patients with chronic interstitial nephropathy living in an area of Tunisia known to have high dietary exposure to ochratoxin A (Hassen et al., 2004).

2.3.3 Epidemiological studies

The earlier literature on the association between human exposure to ochratoxin A via the diet and the occurrence of Balkan endemic nephropathy and associated urinary tract tumours was summarized by the Committee in its previous evaluation (Annex 1, reference 153). However, contrary to the clear causal evidence of ochratoxin A-induced nephrotoxicity and nephrocarcinogenicity in rodents (Annex 1, reference 153; O'Brien & Dietrich, 2005; European Food Safety Authority, 2006), the significance of ochratoxin A for human health still remains unclear from the available epidemiological evidence (Fink-Gremmels, 2005; European Food Safety Authority, 2006). Moreover, ochratoxin A exposure is only one of several hypotheses concerning an environmental etiology for Balkan endemic nephropathy; exposure to aristolochic acid from the seeds of a weed (*Aristolochia clematis*) that contaminates wheat in endemic regions and intake of potentially carcinogenic organic compounds via drinking-water from the occurrence of lignites from Pliocene-age coals in endemic regions have also been considered as potential causes of Balkan endemic nephropathy and urinary tract tumours (Stefanovic et al., 2006).

Kidney function in infants breastfed for at least 4 months has been assessed in relation to maternal and infant serum and maternal milk concentrations of ochratoxin A. Thirty-six (72%) of the 50 mothers included in the study had detectable ochratoxin A in serum and milk. Infants with higher ochratoxin A levels in their serum (≥ 2 ng/ml) had higher levels of urinary β 2-microglobulin and microalbuminuria than did infants with lower serum levels (< 2 ng/ml). Both differences were statistically significant with the use of univariate analysis. Multivariate logistic regression analysis showed that there was a significant correlation between a higher infant serum level of ochratoxin A and the degree of microalbuminuria, but not β 2-microglobulinuria (Hassan et al., 2006).

The possible role of a genetic predisposition to toxicity from ochratoxin A has been investigated in Jelma, Tunisia, an area in which it is known that there is high dietary exposure to ochratoxin A and in which chronic interstitial nephropathy of unknown etiology with similarities to Balkan endemic nephropathy is often seen. Twenty-one people in four rural families, all exposed to ochratoxin A via the diet, were tested for urinary β 2-microglobulin concentration, ochratoxin A concentration in blood, urine and food and human histocompatibility leukocyte antigen (HLA) haplotype. Ochratoxin A was detectable in blood and urine in 19 out of the 21 persons at concentrations in blood ranging from 8 to 1468 ng/ml. Three siblings in

one family had chronic interstitial nephropathy (altered renal function, β_2 -microglobulinuria and renal karyomegaly confirmed by histology). Haplotyping showed that the three with chronic interstitial nephropathy shared elements of the phenotype A3, B27/35 and DR7 (Creppy et al., 2005).

Familial clustering of Balkan endemic nephropathy has prompted a number of other genetic investigations. Limited studies on genetic polymorphisms in xenobiotic metabolizing enzymes in Bulgarian Balkan endemic nephropathy patients and their healthy relatives have shown a significantly higher risk for Balkan endemic nephropathy in individuals carrying the CYP3A5*1 allele, but the possible role of CYP3A5 activity in the development of Balkan endemic nephropathy is not known. A higher frequency of *GSTM1* wild-type allele (conferring a high activity of GSH transferases) has also been found in Balkan endemic nephropathy patients compared with controls, while the Multidrug Resistance 1 (*MDR1*) haplotype is associated with a lower frequency of Balkan endemic nephropathy (Stefanovic et al., 2006).

3. ANALYTICAL METHODS

Ochratoxin A is a colourless crystalline compound with blue fluorescence under ultraviolet light. It has a weak acidic character, with pK_a values of 4.4 (carboxyl group of phenylalanine) and approximately 7.5 (phenolic group). Acid hydrolysis splits ochratoxin A into phenylalanine and ochratoxin *alpha*, an optically active lactone acid. The lactone ring may be opened by alkali or treatment with some microbial esterases, and this removes toxicity. This reaction is reversible, under acidic conditions (Monaci & Palmisano, 2004).

Methods for analysing ochratoxin A were thoroughly reviewed in the previous report (Annex 1, reference 153). At that time, it was reported that validated analytical methods were available for the determination of ochratoxin A in maize, barley, rye, wheat, wheat bran, wholemeal wheat, roasted coffee, wine and beer. The best methods used LC following cleanup using immunoaffinity columns. Over 40 methods were reviewed. This review is limited to recent developments.

3.1 Sample preparation

Sample preparation may involve either a milling or a wet slurry technique. A recent study has evaluated these two processes using 10-kg samples of cocoa, green coffee, almonds and pistachios and has shown that the wet slurry process has a lower coefficient of variation than dry milling (Spanjer et al., 2006). The investigators concluded that this was due to the smaller particle size produced by the wet slurry process and that this method of sample preparation was to be preferred.

3.2 Extraction

Traditionally, extraction has been by conventional liquid-liquid methods (Annex 1, reference 153). Recently, a number of methods for solid-state extractions have been published. These typically use derivatized silica with C_8 , C_{18} and

cyanopropyl (CN) stationary phases (Monaci & Palmisano, 2004). In a typical solid-phase extraction, column conditioning is followed by application of the liquid sample. Most interferences can be removed by a washing step. The analyte is then eluted with an organic solvent and, after drying, dissolved in a small amount of the mobile phase (Monaci & Palmisano, 2004). This procedure is relatively non-selective, and the cleanup levels achieved may be insufficient for some of the more challenging matrices that commonly contain ochratoxin A, including coffee, beer and wine. Antibody-based immunoaffinity columns are commonly used after solid-state cleanup for samples of these commodities.

For many solid samples, a solvent extraction step is still necessary. Extraction with acidic chloroform, acidic ethyl acetate containing 2 mol sodium chloride/l or methanol–sodium carbonate (1%), followed by back-extraction to sodium bicarbonate, has been recommended (Monaci & Palmisano, 2004). After washing the column, ochratoxin A can be eluted with methanol and, if necessary, further purified through an immunoaffinity column.

Immunoaffinity columns are expensive, and recently the use of more stable imprinted polymers has been described. With careful design, imprinted polymers have been reported to be very promising and cheaper alternatives to both solid-phase extraction and immunoaffinity columns (Monaci & Palmisano, 2004).

3.3 *Ochratoxin A estimation*

High-performance liquid chromatography (HPLC) remains the most popular method for determination of ochratoxin A. Most HPLC methods use a reversed-phase column and an acidic mobile phase, so the carboxyl group of the mycotoxin is in the undissociated form. The post-column addition of 10% ammonia solution increases the fluorescence emission of ochratoxin A by a factor of 10 (Monaci & Palmisano, 2004).

Recent advances have included the coupling of HPLC to a mass spectrometer using electrospray ionization (Timperio et al., 2006; Ventura et al., 2006).

TLC is still regarded as a cheap and effective technique for ochratoxin A estimation, particularly because of its low cost and adaptability. New methods include extraction with a mixture of phosphoric acid and dichloromethane and purification by liquid–liquid partitioning into sodium hydrogen carbonate, before separation by normal-phase TLC and detection by fluorescence as usual (Pittet & Royer, 2002); and extraction with a mixture of methanol and aqueous sodium bicarbonate solution, followed by partitioning into toluene before TLC (Ventura et al., 2005).

A number of recent papers deal with immunoassay techniques, especially various enzyme-linked immunosorbent assays (ELISA). Detection limits for HPLC and ELISA are quite comparable, but the ELISA technique suffers from false-positive results, because of cross-reactions, and, more importantly, false-negative results, especially from tissue extracts (Monaci & Palmisano, 2004).

Validation of mycotoxin methods has become very important, because they are used for regulatory purposes throughout the world. A list of known validated or official methods for ochratoxin A analysis is given in Table 7.

Table 7. Validated and official analytical methods for determining ochratoxin A in food samples^a

Food	Cleanup	Recovery (%)	RSD (%) within lab	RSD (%) between labs	Reference
Baby food	IAC	108	18–36	29–63	Burdaspal et al. (2001)
White wine	IAC	88–105	7–11	13–16	Visconti et al. (2001)
Red wine	IAC	84–93	7–11	14–14	Visconti et al. (2001)
Beer	IAC	87–95	5–17	15–26	Visconti et al. (2001)
Roasted coffee	SPE + IAC	65–97	2–22	14–26	Entwisle et al. (2001)
Barley	IAC	65–113	4–24	12–33	Entwisle et al. (2000)
Barley	CC	64–72	21	23–28	Larsson & Moller (1996)
Wheat	IAC	91	10–13	14–32	Scott (2002)
Wheat bran	CC	64–72	17	20–28	Larsson & Moller (1996)
Rye	CC	64–72	12	18–31	Larsson & Moller (1996)
Maize	CC	53–97	20	21–32	Nesheim et al. (1992)
Pig kidney tissue	CC	53–97	16	33–68	Nesheim et al. (1992)
Cocoa	IAC	–	–	15–40	Brera et al. (2005)

CC, column chromatography; IAC, immunoaffinity column; RSD, relative standard deviation; SPE, solid-phase extraction.

^a Modified from Monaci & Palmisano (2004).

4. SAMPLING PROTOCOLS

Sampling for analysis is always very important, but often neglected. Recently, Vargas et al. (2006a, 2006b, 2006c) published extensive information on the parameters governing sample size for testing green coffee for ochratoxin A.

5. LEVELS AND PATTERNS OF CONTAMINATION OF FOOD COMMODITIES

At the fifth-sixth meeting of JECFA (Annex 1, reference 152), it was reported that ochratoxin A is produced by a single *Penicillium* species, *P. verrucosum*, by *Aspergillus ochraceus* and several related *Aspergillus* species, and by *A. carbonarius*, with a small percentage of isolates of the closely related species *A.*

niger. Since that report, some other *Aspergillus* and *Penicillium* species have been described as potential sources of ochratoxin A. One addition is that of *Penicillium nordicum*, recognized as a second *Penicillium* species able to produce ochratoxin A (Larsen et al., 2001). The latest information also indicates that *A. ochraceus* is an uncommon fungus, and isolates do not often produce ochratoxin A. However, two recently described species, *A. westerdijkiae* and *A. steynii*, split off from *A. ochraceus*, are the major producers. The following sections deal with these species in some detail.

5.1 *Aspergillus* species that produce ochratoxin A

Aspergillus species producing ochratoxin A can be divided into two distinct groups. The first includes species producing conidia coloured golden brown, classified in *Aspergillus* section *Circumdati* and related to *A. ochraceus*. The second includes species producing black conidia, classified in *Aspergillus* section *Nigri*, of which *A. carbonarius* is the most important.

5.1.1 *Species in Aspergillus* section *Circumdati*

Ten species classified in section *Circumdati* were reliably reported to produce ochratoxin A by Frisvad et al. (2004b). Most are rare in nature or at least uncommon in foods and not likely to contribute to the level of ochratoxin A in the diet. *Aspergillus cretensis*, *A. flocculosus*, *A. pseudoelegans*, *A. roseoglobosus*, *A. sulphureus* and *A. muricatus* (sexual state *Neopetromyces muricatus*) all fit into that category. *Aspergillus alliaceus* (sexual state *Petromyces alliaceus*), not usually classified in section *Circumdati*, but morphologically belonging there, also produces ochratoxin A. It is not found in foods.

Aspergillus sclerotiorum is a somewhat more common species in foods, but isolates produce only low levels of ochratoxin A (Frisvad et al., 2004b). *Aspergillus ochraceus*, traditionally regarded as a common species with rather variable morphology, has recently been divided into three species. *Aspergillus ochraceus* sensu stricto is now regarded by Frisvad et al. (2004b) as rather uncommon. Its role in that restricted sense may or may not be important in human health terms. Two recent segregates from *A. ochraceus* are now considered to be the major species in *Aspergillus* section *Circumdati* contributing ochratoxin A to the human diet.

The first of these species is *A. westerdijkiae*; according to Frisvad et al. (2004b), this is the correct name for the original "*A. ochraceus*" from South Africa that led to the discovery of ochratoxins. *Aspergillus westerdijkiae* differs from *A. ochraceus* by its inability to grow at 37 °C and by producing white to cream sclerotia rather than the purple ones of *A. ochraceus* (Frisvad et al., 2004b). *Aspergillus westerdijkiae* is a common species and a high producer of ochratoxin A (Frisvad et al., 2004b). The second new species, *A. steynii*, also differs from *A. ochraceus* by its inability to grow at 37 °C and from *P. westerdijkiae* by producing ellipsoidal, not spherical, conidia—and from both species by producing pale yellow, not golden brown or ochre, conidia (Frisvad et al., 2004b). Most *P. steynii* isolates produce large amounts of ochratoxin A, and it is a quite commonly occurring species (Frisvad et al., 2004b).

Much biochemical work reportedly carried out on *A. ochraceus* has really been on *A. westerdijkiae* (Frisvad et al., 2004b), but these three species are sufficiently similar that that work retains its value. The name *A. ochraceus* is likely to continue to be used for all three of these species in many reports in the future.

5.1.2 *Species in Aspergillus section Nigri*

Samson et al. (2004) reported that four species in *Aspergillus* section *Nigri* (the black *Aspergilli*) are capable of producing ochratoxin A. Two of these species were described as new by those authors. One of these, *A. sclerotiumniger*, is known only from a single isolate. The second, *A. lactiocoffeatus*, is also very uncommon and may represent only a pale colour mutant of *A. niger*. All three known isolates of *A. lactiocoffeatus* produce ochratoxin A, however. The major producer of ochratoxin A among the black *Aspergilli* is now well established to be *A. carbonarius*. This is an old, well known and well defined species, but reports of its ability to produce ochratoxin A first appeared only in 1995 (Horie, 1995). This ability has been substantiated by many authors since (see Samson et al., 2004 for review). Virtually all known isolates of this species produce ochratoxin A.

The fourth ochratoxin A-producing species is *A. niger*, but only a very low percentage (6% or less) of true *A. niger* strains make ochratoxin A (Abarca et al., 2003; Pardo et al., 2004; and many more recent studies; see Samson et al., 2004 for review).

Other black *Aspergilli* of common occurrence in foods are *A. japonicus*, distinguished by microscopic appearance (only phialides are produced), and *A. tubingensis*, distinguished from *A. niger* by molecular methods. Neither of these species makes ochratoxin A (Samson et al., 2004).

5.2 *Penicillium species that produce ochratoxin A*

Since the previous report (Annex 1, reference 153), some isolates previously considered to be *Penicillium verrucosum* have been segregated on various taxonomic grounds into a separate species, *P. nordicum*. Larsen et al. (2001) reported that *P. nordicum* isolates also produce ochratoxin A. *Penicillium verrucosum* and *P. nordicum* are classified in *Penicillium* subgenus *Penicillium* and are very closely related in appearance and physiology. However, while *P. verrucosum* is of common occurrence in cereals from cold climates, *P. nordicum* has been reported to occur in dried proteinaceous foods such as salami, ham and cheese (Frisvad et al., 2004a). On current evidence, *P. nordicum* appears to be a minor source of ochratoxin A in foodstuffs by comparison with *P. verrucosum*.

Although many other *Penicillium* species have been reported to make ochratoxin A, none of these reports is considered to be correct (Frisvad et al., 2004a).

5.3 *Physiology and ecology of fungi that produce ochratoxin A*

The three major groups of fungi producing ochratoxin A have quite different physiology and consequently quite different ecological habitats. To understand the

kinds of foods in which ochratoxin A occurs or to predict the potential for ochratoxin A formation, it is necessary to understand the physiology and ecology of these species and the differences between them. This information is outlined below.

5.3.1 *Penicillium verrucosum* and *P. nordicum*

Penicillium verrucosum is a slow-growing species under any conditions, but capable of growth at low water activity (a_w) (down to 0.80) and at low temperature (range 0–31 °C, optimum 20 °C) (Pitt & Hocking, 1997). The physiology of *P. nordicum* has been little studied, but, as it is closely related to *P. verrucosum*, it can be expected to have similar physiological characteristics. In particular, it grows poorly, if at all, at 30 °C, like *P. verrucosum* (Samson & Frisvad, 2004).

A notable feature of the ecology of *P. verrucosum* is that it grows only at lower temperatures. This results in a distribution that is apparently confined to cool temperate regions. Cereal crops are its major food habitat, and it ranges across northern and central Europe and Canada. It appears to be uncommon, indeed almost unknown, in warm climates or in other kinds of foods. The occurrence of this species in European cereals has two consequences: ochratoxin A is present in many kinds of European cereal products, especially bread and flour-based foods, and it is present in animals that eat cereals as a major dietary component. Ochratoxin A was detected in Danish pig meats 25 years ago (Krogh et al., 1973), and its implications for human and animal health were recognized at the same time. As bread and other cereal products and pig meats are major components of the European diet, the further consequence is that most Europeans who have been tested have shown appreciable concentrations of ochratoxin A in their blood (Hald, 1991; and many more recent reports). There is no doubt that this results from the growth of *P. verrucosum* in cereals.

In studying the mycoflora of grapes used for the production of a particular kind of dessert wine, Torelli et al. (2006) reported on the identification of 379 strains of *Penicillium* species isolated from the grapes. None was *P. verrucosum* or *P. nordicum*; this report reinforces the belief that *Penicillium* species are not responsible for the presence of ochratoxin A in grapes or grape products.

Penicillium nordicum has been reported to occur in meat products (salami, ham, sausage, chicken meat) and in cheese (Samson & Frisvad, 2004). It appears to be much more widely distributed than *P. verrucosum*, but nearly all reported sources have been products kept under refrigeration during manufacture. There have been few studies on its ecology. However, during a 1-year survey of seven ham manufacturing plants in Europe, Battilani et al. (2007) isolated potential mycotoxin-producing fungi from factory air and from the surface of the curing hams. More than 90% of the 19 000 fungal isolates examined were *Penicillium* species. When 300 of these isolates, randomly sampled, were identified to species, 10% were *P. nordicum*. When these 30 isolates were tested for ochratoxin A production on laboratory media, 50% were positive. Escher et al. (1973) had reported ochratoxin production on dry cured hams; an explanation is suggested by Battilani et al. (2007).

5.3.2 *Aspergillus ochraceus* and close relatives

Aspergillus ochraceus can be described as a mesophilic xerophile. Growth occurs between 8 °C and approximately 40 °C, with the optimum at 24–31 °C (Pitt & Hocking, 1997). Optimal conditions for growth are 0.95–0.99 a_w , whereas the lower limit for growth is 0.79 a_w on media containing sugars and down to 0.81 a_w on media based on sodium chloride. *Aspergillus ochraceus* grows slowly at pH 2.2 and well between pH 3 and 10 (Pitt & Hocking, 1997). Little is known about the physiology of *A. westerdijkiae* or *P. steynii*, except that they do not grow at 37 °C (Frisvad et al., 2004b). Other parameters governing growth can be expected to be similar to those for *A. ochraceus*. Indeed, some published data on *A. ochraceus* physiology have certainly used strains now classified as one of these two species (Frisvad et al., 2004b).

Because *A. westerdijkiae* and *A. steynii* have been distinguished from *A. ochraceus* only recently, the reported ecology of *A. ochraceus* is actually that of these three species. They have been isolated from a wide range of food products, but are more common in dried and stored foods than elsewhere. Stored foods from which these species (reported as *A. ochraceus*) have been isolated include smoked or salted dried fish, dried beans, biltong, soybeans, chickpeas, rapeseed, pepper, dried fruit and sesame seeds. Nuts are also a major source, especially pecans and pistachios, as well as peanuts, hazelnuts and walnuts. Reports from cereals and cereal products have been infrequent, but records include rice, barley, maize, wheat, flour and bran. These species have also been reported from cheese, spices, black olives, cassava and processed meats. However, none of these species is a common cause of food spoilage, so their presence is not a good indicator of significant mycotoxin production (Pitt & Hocking, 1997).

Numerous studies have detected *A. ochraceus* (and related species) in green coffee beans (e.g. Levi et al., 1974; Cantafora et al., 1983; Studer-Rohr et al., 1994; Taniwaki et al., 2003). Although these species are one source of ochratoxin A in coffee (Taniwaki et al., 1999), studies indicate that *A. carbonarius* is a more important source in coffee from most producing countries.

Aspergillus ochraceus has been isolated from a variety of Southeast Asian commodities, including maize, peanuts, soybeans and other beans, cashews and sorghum. Its presence or absence in any sample probably was related to length of storage rather than to geographical location or other factors (Pitt et al., 1993, 1994, 1998).

5.3.3 *Aspergillus carbonarius* and *A. niger*

The ability of *A. carbonarius* to produce ochratoxin A was first reported only 12 years ago (Horie, 1995; Téren et al., 1996; Varga et al., 1996; Heenan et al., 1998), but its importance is now well recognized. This species resembles *A. niger* in many features, and indeed the two species are very closely related. *Aspergillus carbonarius* differs from *A. niger* most notably in the production of larger spores, although other minor morphological differences exist. *Aspergillus carbonarius* grows at rather lower temperatures than *A. niger*, with a maximum around 40 °C and optimal conditions at 30 °C (Leong et al., 2006a; Romero et al., 2007). The

ability to grow at reduced a_w is also more restricted: germination occurs down to 0.85 a_w at 25 and 30 °C (Romero et al., 2007). Maximum ochratoxin A production has been reported to occur at 15 °C and 0.95–0.98 a_w (Estaban et al., 2006a; Leong et al., 2006a). Little ochratoxin A was produced above 25 °C (Leong et al., 2006a). Ochratoxin A was produced over a very wide pH range of 2 or 3–10 (Estaban et al., 2006b).

Aspergillus niger grows optimally at relatively high temperatures, with a growth minimum of 6–8 °C, a maximum of 45–47 °C and optimal conditions at 35–37 °C. *Aspergillus niger* is a xerophile, with germination reported at 0.77 a_w at 35 °C. Growth rates vary only slightly on media based on sugars, sodium chloride or glycerol or on media of pH 4.0 and 6.5, at various water activities; in other words, growth of *A. niger* appears to be little affected by food type. *Aspergillus niger* is able to grow down to pH 2.0 at high a_w (Pitt & Hocking, 1997).

Several other black *Aspergillus* species commonly occur in foods, especially *A. tubingensis* and *A. japonicus*, but these species do not produce ochratoxin A. Their physiology appears to be broadly similar to that of *A. niger*.

Aspergillus carbonarius, *A. niger* and *A. japonicus* occur together in foods and superficially look similar, so many surveys of Aspergilli in foods have not differentiated these three species, calling all black Aspergilli *A. niger*.

The black *Aspergillus* species have a high resistance to sunlight and ultraviolet light (Rotem & Aust, 1991; Leong et al., 2006b) and grow at relatively high temperatures. They are also very acid tolerant and prefer a somewhat reduced water activity (Leong et al., 2006a). For all these reasons, a major habitat for the black Aspergilli is in grapes (Snowdon, 1990), but it has not been shown that *Aspergillus* species are primary pathogens. The evidence is that they are able to invade only damaged grapes (Snowdon, 1990). Damage can be caused by the growth of pathogenic fungi such as *Botrytis cinerea*, *Rhizopus stolonifer* or powdery mildew or from skin splitting due to rain damage before harvest. Inclusion of damaged grapes at harvest or delays in crushing, conditions that permit continued growth of the black Aspergilli, mean that wines frequently contain low levels of ochratoxin A. Toxin production ceases at the time oxygen is removed from the must at the start of fermentation.

Where grapes are further processed to make dried vine fruits, the black Aspergilli continue to grow, as grapes are dried in the sun without preservatives in most producing countries. Dried grapes (raisins, sultanas) can therefore readily contain unacceptable levels of ochratoxin A (Lombaert et al., 2004; Iamanaka et al., 2005; Jorgensen, 2005; Meyvaci et al., 2005).

Many studies (e.g. Abarca et al., 2003 and many later papers) have now shown that *A. carbonarius* is the major source of ochratoxin A in wines and other grape products throughout the world. The incidence of fruit damage, grape splitting and high temperatures at harvest, together with poor harvest practice such as delayed crushing in wine making, all contribute to the level of ochratoxin A in wines and dried grape products.

The previous assessment (Annex 1, reference 153) concluded that contamination of cereals with ochratoxin A occurs only in cool temperate zones, as the result of invasion by *P. verrucosum*. Although ochratoxin A was occasionally found in long-stored cereals, owing to the presence of *A. ochraceus* (or one of its close relatives), this is an uncommon occurrence (Pitt & Hocking, 1997). However, with the recent identification of *A. carbonarius* as a source of ochratoxin A in foods, the reported presence of ochratoxin A in tropical cereals from time to time can now be explained readily. It has been known for some years that *A. niger* commonly occurs in tropical cereals (Pitt et al., 1993, 1998), and all the evidence indicates that *A. carbonarius* will also be present under these conditions.

Aspergillus niger is by far the most common *Aspergillus* species responsible for post-harvest decay of fresh fruit, including apples, pears, peaches, citrus, grapes, figs, strawberries, mangoes, tomatoes and melons and some vegetables, especially onions, garlic and yams (Snowdon, 1990, 1991). It is not clear whether *A. carbonarius* occurs along with *A. niger* on these fresh foods. Most of these diseases are sporadic and of minor significance. Except where fruits are used for processing, rots due to *A. niger* are not considered a public health problem, as mouldy fruit is normally discarded.

Aspergillus niger is very frequently isolated from sun-dried products, such as vine fruits, dried, smoked and cured fish, biltong, cocoa beans and spices. It sometimes causes kernel rot in cashews and is one species causing thread mould spoilage of cheese (Hocking & Faedo, 1992).

Aspergillus niger is among the most common fungi isolated from nuts, especially peanuts, and has also been reported from pecans, pistachios, hazelnuts, walnuts, coconut and copra. Cereals and oilseeds are also sources, especially maize and also barley, soybeans, canola, sorghum, stored and parboiled rice and dried beans (Pitt & Hocking, 1997).

Aspergillus niger was commonly isolated during studies of Southeast Asian foods (Pitt et al., 1993, 1994, 1998). The highest contamination occurred in peanuts, maize, cashews, copra, pepper and spices from Indonesia, Thailand and the Philippines and in kemiri nuts from Indonesia.

In terms of mycotoxin production, *A. niger* is usually regarded as a benign fungus and has been widely used in food processing. It is categorized as "generally regarded as safe" by the government in the United States of America (USA). However, a low percentage (6%; Pardo et al., 2004) of *A. niger* isolates are believed to be able to produce ochratoxin A. Ochratoxin A production by *A. niger* in commercially grown crops appears possible, but is probably uncommon.

6. EFFECTS OF PROCESSING

A few recent studies have examined the effect of processing on concentrations of ochratoxin A in wines, coffee and European cereals.

6.1 Wines

Studies have shown that vinification consistently reduces ochratoxin A concentrations in wine, independent of the initial ochratoxin A concentration in grapes. Two independent studies produced ochratoxin in musts by inoculation of grapes on the vine with spores of *A. carbonarius*. In one study, the mean retention of ochratoxin A from grapes to wine was 8.1% after malolactic fermentation, without the use of fining agents (Fernandes et al., 2007). In a second study, the reduction of ochratoxin A during vinification was followed throughout the process, with the establishment of a mass balance for toxin loss (Leong et al., 2006a). Ochratoxin A was lost at each solid-liquid separation stage of the process. The ochratoxin A concentrations in white and red wines after racking were 4% and 9%, respectively, of those in crushed grapes. Those corresponded to 1% and 6% of the concentrations in the inoculated grapes (Leong et al., 2006a). The mass balance showed that ochratoxin A was not transformed either chemically or biologically by yeast during fermentation, but was discarded with the marc and lees. The greater retention of ochratoxin A in red wines was noted after two processes, pressing and racking. This was attributed to the fact that white grapes are pressed before fermentation, so (in the absence of alcohol) less ochratoxin A partitions into the juice; as a result, more is present in the grape solids and removed during clarification (Leong et al., 2006a). The addition of bentonite at 2.5 g/l to a semillon wine resulted in a 67% reduction in ochratoxin A concentration (Leong et al., 2006e). Addition of yeast hulls at 5.0 g/l caused a 43% reduction in ochratoxin A. Potassium caseinate and gelatin also had some effect on ochratoxin A concentrations, whereas a range of other fining agents had no effect. Storage of wines from 10 to 14 months resulted in decreases in ochratoxin A concentration of 22% and 29% for white and red wines, respectively (Leong et al., 2006d).

Another study examined the performance of 20 strains of *Saccharomyces* in removing naturally present ochratoxin A during vinification. Considerable strain-to-strain variation was observed: residual ochratoxin A ranged from 10% to 60%. When musts were fortified with additional ochratoxin A, residual ochratoxin A ranged from 17% to 34%. The authors concluded that the yeast cell walls were responsible for much of the reduction in ochratoxin A (Caridi et al., 2006).

All of these studies indicate that vinification results in large reductions of ochratoxin A during processing, although the percentage reduction varied considerably between the studies.

6.2 Coffee

A number of studies have looked at the influence of roasting on the ochratoxin A level in coffee. There is general agreement that roasting does have a big influence on ochratoxin A levels, but the percentage reduction reported has varied widely. The temperature of roasting appears to be the major factor influencing results. Using a roasting air temperature of 450 °C, three roasting times were studied by Romani et al. (2003) that resulted in final coffee temperatures of 175, 185 and 204 °C. The light roast (175 °C) caused no reduction in ochratoxin A in one sample, but reductions of 60–80% in three others. The dark roast (204 °C) caused

reductions of more than 90% in all samples. This process was equivalent to a typical espresso coffee brew.

Using artificially contaminated coffee beans (30 µg ochratoxin A/kg), Nehad et al. (2005) reported that roasting reduced ochratoxin A by 31%, that filtering reduced ochratoxin A by 72% and that preparation of Turkish coffee reduced ochratoxin A by 88%.

Pérez de Obanos et al. (2005) analysed nine samples of naturally contaminated Vietnamese coffee. Roasting for 5 min with air at 260 °C produced reductions ranging from 12% to 93%. Preparation of espresso coffee from these roasted samples produced a further reduction of 16–70% in ochratoxin A, whereas the production of mocha coffee caused reductions ranging from 17% to 55%, and the production of filtered coffee caused reductions that ranged from only 1.2% to 25%.

It can be concluded that coffee roasting reduces ochratoxin A in coffee, but the reductions are variable and not fully predictable.

6.3 Milling and breadmaking

Batches of whole wheat contaminated with ochratoxin A were produced by inoculation with *P. verrucosum* under controlled conditions in the laboratory. The fate of ochratoxin A was followed through cleaning, abrasive scouring of the outer grain coat, milling into wholemeal and milling into further fractions. Bread was baked from both wholemeal flour and straight-run white flour. Scouring removed up to 44% of the ochratoxin A present in the wheat, and only a small further loss occurred during the breadmaking process. Using a combination of cleaning, scouring and removal of the bran and offal fractions, an overall reduction of about 75% of ochratoxin A could be achieved in the white bread (Scudamore et al., 2003).

6.4 Extrusion

The extrusion of wholemeal wheat contaminated with ochratoxin A resulted in losses of no more than 40% of the toxin, even under the harshest conditions likely to be used in commercial practice (Scudamore et al., 2004; Castells et al., 2005). This reduction is much lower than had previously been reported for fumonisins, aflatoxins and zearalenone (Castells et al., 2005).

7. PREVENTION AND CONTROL OF OCHRATOXIN A PRODUCTION

The principal fungi that produce ochratoxin A in foods—*Aspergillus westerdijkiae* and *A. steynii* (formerly included in *A. ochraceus*), *A. carbonarius*, *Penicillium verrucosum* and *P. nordicum*—are not associated with plants and hence are not usually present in food crops before harvest. The control of ochratoxin A in foods, therefore, is basically a food technology problem. This means that the basic concepts of good harvest practice, of drying crops rapidly and keeping them dry in storage, transport and processing systems, will ensure that crops remain essentially free of ochratoxins (Pitt, 2006). There is one exception, and this is the possibility

of the entry of *A. carbonarius* into grapes before harvest. Each of the various commodity–fungus combinations will be dealt with in turn below, with the slightly more complicated situation of grapes first.

7.1 *Grapes, wine and other grape products*

Aspergillus carbonarius is not a pathogenic fungus in plants, and there is no evidence that it can invade healthy, intact grapes. However, various factors can cause damage to the skins of grapes before harvest, permitting the entry of this fungus. True grape pathogens, *Botrytis cinerea*, *Rhizopus stolonifer* and especially the powdery mildews, commonly infect grape bunches before harvest (Emmett et al., 1992; Leong et al., 2007). Effective control of these pathogens is very difficult, although reducing leaf numbers to increase sunlight on tight bunches and judicious spray programmes will help. In some cultivars, rain before harvest can cause skin splitting, and this also provides an entry point for *A. carbonarius* (Leong et al., 2004). Once *A. carbonarius* has gained entry to a grape, the high sugar/high acid combination provides a perfect medium for ochratoxin A production.

The keys to low ochratoxin A formation in wine, therefore, are the reliance on prevention of infection by pathogens, rapid harvest if rain causes grape splitting, good harvest practice, including rejection of poor quality bunches, and a minimal delay between harvest and crushing. Crushing rapidly leads to anaerobic conditions, where *A. carbonarius* can no longer grow and ochratoxin A formation ceases.

Control of ochratoxin A formation in dried vine fruits is less easy, because any preharvest infection with *A. carbonarius* will continue to develop during the early stages of drying. In addition, any damage to grapes during harvest and the commencement of drying can lead to new *A. carbonarius* infections. Although the hot, high sunlight levels that occur during drying are not favourable to the growth of *A. carbonarius*, this fungus is less inhibited than almost any other species, and so ochratoxin A production can continue until the water activity of the drying grapes has been reduced substantially (Leong et al., 2006c).

7.2 *Coffee*

Aspergillus westerdijkiae and *A. steynii* (formerly included in *A. ochraceus*) and *A. carbonarius* are the major causes of ochratoxin production in green coffee. There is no evidence that these fungi invade coffee cherries before harvest. Infection occurs during handling after harvest and in the drying yard (Taniwaki et al., 2003).

Coffee plants originated in the highlands of Ethiopia and must be exposed to temperatures below 19 °C in order for flowering to occur. However, berry maturation requires much higher temperatures, so world coffee production occurs in tropical highland regions. In consequence, many coffee-growing areas in the world are subjected to misty or rainy conditions after harvest, with consequent slow drying of the coffee cherries, providing the time needed for these fungi to invade and produce ochratoxin A. However, good processing and drying regimes can prevent this problem. It has been shown that rapid and efficient drying of coffee

cherries after harvest leads to the production of coffee beans free of ochratoxin A (Bucheli & Taniwaki, 2002).

The theory of producing coffee free of ochratoxin is clear, but it will be some time before practical measures can be put in place in all growing areas.

7.3 Cereals in temperate zones

Ochratoxin A in temperate zone cereals is produced by the growth of *P. verrucosum*. Again, there is no evidence that these fungi infect growing cereal plants or occur in nature in grains before harvest. The key to controlling ochratoxin A in cereals is again good drying practice. The theory is clear; however, in cool temperate zones, grain is often harvested during moist or rainy conditions, and control may be difficult in practice. A European Union (EU) project recently examined all aspects of this problem and issued a major report (Olsen et al., 2004).

7.4 Cereals in tropical zones

The crop most at risk from ochratoxin A production in the tropics is maize, as the black *Aspergilli* are commonly present in maize grains, but much less common in other cereals (Pitt et al., 1993, 1994, 1998). Ochratoxin production in maize was not considered to be a problem until quite recently, and in general terms it is much less significant than production of aflatoxins or fumonisins. It is most likely that *A. carbonarius* is the fungus responsible for ochratoxin production in tropical crops such as maize, but this topic has been little researched. *Aspergillus carbonarius* was not distinguished from *A. niger* by Pitt et al. (1993, 1994, 1998). Good drying and storage practice should lead to control of ochratoxin in tropical crops such as maize.

7.5 Meat products

If meat products are infected by *P. nordicum*, this must occur during the processing stages. Control of levels of fungal spores in the air will reduce this problem.

8. FOOD CONSUMPTION AND DIETARY EXPOSURE ASSESSMENTS

The request from CCFAC (Codex Alimentarius Commission, 2006) was to review the available data on the contamination of cereals by ochratoxin A with a particular focus on cereals from developing countries and to assess the possible impact of MLs of 5 and 20 µg/kg for cereals. In addition, the potential impact of new data on the previous exposure assessment done by the Committee was estimated.

8.1 Analysis of data submitted

Data on the occurrence of ochratoxin A in cereals were submitted by Canada, Germany, Japan and the EU. In addition, data reporting the contamination of cereals in Africa (Nigeria, Ghana and Burkina Faso) were submitted jointly by the

Food and Agriculture Organization of the United Nations (FAO) and Consiglio Nazionale delle Ricerche (CNR), Italy, and information regarding the contamination of cocoa and coffee beans was submitted by Côte d'Ivoire. Table 8 describes the various aspects of the distribution of ochratoxin A contamination in cereals.

8.1.1 Canada

Canada submitted a probabilistic exposure assessment based on occurrence data for ochratoxin A in cereals and other food commodities and on food consumption data from the USA. Detailed individual data on occurrence were not submitted, but ochratoxin A was detected in 436 samples out of 1668 analytical results.

Table 8. Description of data submitted for this evaluation: distribution of ochratoxin A contamination in cereals

Countries	Number of samples	Number of non-detected ^a (%)	Samples >LOD or LOQ and <5 µg/kg (%)	Samples ≥5 µg/kg (%)	Samples >10 µg/kg (%)	Samples >15 µg/kg (%)	Samples >20 µg/kg (%)
Germany (RC)	1719	1428 (83)	250 (15)	18 (1.1)	8 (0.5)	6 (0.4)	6 (0.4)
Germany (PC)	2377	1717 (72)	639 (27)	21 (0.9)	11 (0.5)	5 (0.2)	2 (0.1)
Japan	197	188 (95)	9 (5)	0	0	0	0
Africa	27	9 (33)	16 (59)	2	0	0	0
Canada (RC)	1016	827 (82)	—	—	—	—	—
Canada (PC)	652	405 (62)	—	—	—	—	—
EU (RC + PC)	5117	2295 (45)	2779 (54)	43 (0.8)	17 (0.3)	—	10 (0.2)

LOD, limit of detection; LOQ, limit of quantification; RC, raw cereals; PC, processed cereals.

^a Non-detected samples are those for which no numerical value was provided. They can be either results below the LOD or in certain cases results between the LOD and the LOQ.

The report provides various estimations of the average contamination level, replacing the left-censored data by half of the limit of detection (LOD) or half of the limit of quantification (LOQ) when the LOD was not provided (scenario 1) or applying a parametric model assuming the lognormality of the distribution (scenario 2). The authors also truncated the distribution assuming that an ML was implemented and fully enforced (scenario 3) and compared the resulting mean concentration with a distribution not censored on the right (scenario 4). Interestingly, none of the scenarios shows a significant impact on the average concentration for each lot analysed. However, the observed differences in the mean contaminations are

obviously driven by the LOD and LOQ, because for samples analysed by Health Canada, the LOD was 0.05 µg/kg for most of the cereals, whereas for the data analysed by the Canadian Grain Commission, the LOD was not available and an LOQ of 1 µg/kg was used to calculate the average contamination level.

For the assessment of dietary exposure, the authors used the full distribution of the contamination for all commodities combined with the frequencies and amounts consumed and estimated an average dietary exposure below 5 ng/kg bw and high percentiles of exposure below 15 ng/kg bw. Because of the methodological choice of a probabilistic exposure assessment, cut-off points on the right of the distribution curve to simulate enforced MLs have an impact on dietary exposure when the cut-off points are located in the tail (e.g. at the level of 5 µg/kg). On the contrary, a cut-off point at a level above most of the observed values of contamination (e.g. 20 µg/kg) is ineffective.

8.1.2 Germany

Germany submitted 4097 individual analytical results for ochratoxin A collected between 1999 and 2006 in different cereals and foodstuffs derived from cereals. Data are described in [Table 8](#).

The assays were distributed as follows:

- 624 samples of wheat and 580 samples of wheat flour and other wheat products;
- 414 samples of rye and 368 samples of rye flour and other rye products;
- 197 samples of barley and 16 samples of barley products;
- 30 samples of oat and 249 samples of oat flakes and other oat products;
- 19 samples of maize and 221 samples of maize meal, cornflakes and other maize products;
- 285 samples of rice and 21 samples of rice products;
- 30 samples of buckwheat and 18 samples of buckwheat products;
- 499 samples of muesli and muesli products;
- 120 samples of other cereals and 405 samples of products derived from other cereals.

The highest ochratoxin A levels in unprocessed cereals were in rye and buckwheat. Three samples of rye contained a very high concentration of ochratoxin A (95.6–125 µg/kg). A high number (33.3%) of analysed samples of oat contain elevated levels (mean = 0.27 µg/kg).

In those products derived from cereals, the highest ochratoxin A levels were found in products derived from buckwheat (mean = 1.94 µg/kg) and from rye (mean = 0.54 µg/kg).

8.1.3 Japan

Japan submitted individual analytical data for 98 samples of rice and 99 samples of wheat. For rice, only one sample contained ochratoxin A above the LOD (0.08 µg/kg) but below the LOQ. For wheat, seven samples were above the LOD

(0.07 µg/kg) and below the LOQ, and only one sample was above the LOQ, with an ochratoxin A concentration of 0.7 µg/kg.

8.1.4 *European Union*

The European Commission submitted a report compiling 5117 (aggregated) analytical results for ochratoxin A in cereals and cereal products, collected by EU Member States (European Commission, 2002). The total number of positive samples for cereals and cereal products was 55%, and they ranged in concentration from the LOD (0.005 µg/kg) to 33.3 µg/kg. The weighted mean (assuming that the samples in which ochratoxin A was not detected [ND] are contaminated at half of the LOD) was 0.29 µg/kg.

Since information on the extent of contamination in raw materials is very relevant, not only from the health perspective, but also from the economic and commercial points of view, the occurrence data for cereal raw materials (wheat, corn, oat, millet, rye, barley and rice) were reported separately. The following can be noted:

- The weighted mean (assuming that the samples in which ochratoxin A was ND are contaminated at half of the LOD) ranged from 0.136 µg/kg for millet ($n = 34$) to 0.597 µg/kg for rye ($n = 444$).
- Among the individual cereal commodities, wheat has been investigated more widely than the other grains, especially in northern Europe.
- Rye contained the highest ochratoxin A levels, with 50% positive samples.

8.1.5 *Nigeria, Ghana and Burkina Faso*

CNR, in agreement with FAO, provided 27 analytical results on ochratoxin A contamination of cereals in Nigeria, Ghana and Burkina Faso. Ochratoxin A was detected in sorghum, maize and millet samples. Maize had the highest levels of ochratoxin A contamination.

8.2 *Estimation of the content of ochratoxin A in cereals*

8.2.1 *Background*

The critical effects of ochratoxin A relate to long-term exposure. Furthermore, considering that the variability of ochratoxin A concentrations in food depends on climatic and storage conditions, it is unlikely that most consumers would be exposed on a long-term basis to high levels of contamination. Therefore, the central tendency of the distribution of contamination should be combined with food consumption levels for dietary exposure assessments. In addition, owing to the fact that generally more than 50% of analysed samples are below the LOD or LOQ, the use of the median value to represent this central tendency is problematic. Therefore, the international exposure assessments to date are based on the mean level of contamination.

In 2001, the Committee estimated the overall dietary exposure of ochratoxin A to be 43 ng/kg bw per week. The contribution from cereals was estimated to be 58% of the overall exposure (25 ng/kg bw per week), and the level of exposure from food sources other than cereals was therefore equal to 42% of the overall exposure, corresponding to 18 ng/kg bw per week.

In 2006, EFSA evaluated ochratoxin A and estimated a total mean dietary exposure of 15–20 ng/kg bw per week with a high percentile around 60 ng/kg bw per week, based on an average contamination for cereals of 0.29 µg/kg and a high percentile for cereal consumption of 568 g/day (97.5th percentile consumers only in Sweden) (European Food Safety Authority, 2006).

8.2.2 Estimation of the mean contamination for ochratoxin A

African samples were not used for the dietary exposure assessment because they were targeted samples with fungal contamination. Individual data were not available either from Canada or from the EU. Finally, the Committee decided not to merge individual analytical data from Germany and Japan and to focus for the dietary exposure on the largest data set represented by German analytical results for raw and processed cereals.

In order to estimate the mean contamination of ochratoxin A in cereals, a strategy must be applied regarding the replacement of values for samples below the LOD/LOQ. Several approaches can be used, either by replacing the value by 0, LOD/2 or LOD or by modelling the distribution (Verger & Tressou, 2006). For the purpose of the current assessment and considering that more than 60% of samples are not detected, the assignment of ND equal to 0 and ND equal to LOD or LOQ was used to provide a lower- and an upper-bound concentration of ochratoxin A in cereals and to be consistent with World Health Organization (WHO) recommendations.

Analytical results (detected or non-detected) for which information on LOD or LOQ was not available were removed from the data set. In addition, analytical results obtained with methods with high LOQs (i.e. higher than the mean concentration) can artificially influence the estimation of the mean contamination. Therefore, the impact of removing analytical data obtained with the LOQs between 0.5 and up to 3 µg/kg was tested. The Committee concluded that analytical results with an LOQ higher than 1 µg/kg were not fit for the purpose of the current assessment and were removed from the data set. The Committee also considered that removing results obtained with an LOQ between 0.5 and 1 µg/kg would not result in additional improvement of the estimate.

Finally, 1462 and 2070 analytical results were used, respectively, for raw cereals and cereal products. The resulting mean and other main statistical descriptors of the distribution curves are compiled in [Tables 9](#) and [10](#).

Table 9. Ochratoxin A distribution in the German data set on raw cereals (1462 samples): impact of various MLs on the mean concentration of ochratoxin A

	Concentration of ochratoxin A in raw cereals (µg/kg)	
	ND = 0	ND = LOD or LOQ
Distribution of ochratoxin A		
Mean	0.44	0.53
Median	0.00	0.10
90th percentile	0.26	0.50
95th percentile	0.78	0.90
99th percentile	4.98	4.98
Maximum	125.00	125.00
Impact of MLs on mean concentration of ochratoxin A		
ML = 5 µg/kg	0.10	0.19
ML = 20 µg/kg	0.15	0.24
No ML	0.44	0.53

Table 10. Description of ochratoxin A distribution in the German data set on processed cereals (2070 samples): impact of various MLs on the mean concentration of ochratoxin A

	Concentration of ochratoxin A in processed cereals (µg/kg)	
	ND = 0	ND = LOD or LOQ
Distribution of ochratoxin A		
Mean	0.31	0.39
Median	0.00	0.10
90th percentile	0.65	0.70
95th percentile	1.40	1.40
99th percentile	4.73	4.73
Maximum	22.80	22.80

8.2.3 Impact of new data submitted on estimate of mean ochratoxin A concentration

Based on the submission of more than 11 000 samples of cereals and cereal products (individual + aggregated), only 87 of them (0.8%) are contaminated with ochratoxin A at concentrations between 5 and 20 µg/kg, and only 21 samples are contaminated above 20 µg/kg (0.2%); therefore, the theoretical difference between the two MLs of 5 and 20 µg/kg would represent about 0.6% of cereals.

From the German data described above and in Table 9, it is possible to simulate the impact of setting various MLs, assuming 100% enforcement, on mean ochratoxin A concentration by excluding analytical results above each ML. Based

on the available individual analytical results for raw cereals, the mean contamination was calculated using two scenarios for left censorship (i.e. ND = 0 and ND = LOD/LOQ), two scenarios for right censorship (i.e. ML at 5 or 20 µg/kg) and finally a scenario without an ML, resulting in six scenarios compiled in [Table 9](#).

The available data do show a limited impact of setting any of the proposed MLs on the mean contamination of raw cereals by ochratoxin A. However, it is important to note that the observed differences are due to very few analytical results (15 samples out of 1462 excluded when the ML is 5 µg/kg and 6 samples out of 1462 excluded when the ML is 20 µg/kg).

8.2.4 Impact of new data submitted on estimate of dietary exposure

In 2001, the mean overall dietary exposure to ochratoxin A based mainly on European data was estimated by the Committee to be 43 ng/kg bw per week. The contribution from cereals was estimated to be 58% of the overall exposure (25 ng/kg bw per week) based on a daily consumption of 230 g of cereals and a mean concentration of ochratoxin A of 0.94 µg/kg for raw cereals.

The concentrations of ochratoxin A in processed cereals submitted to this Committee are detailed in [Table 10](#). The mean concentration for ochratoxin A ranges from 0.31 µg/kg (lower-bound estimate with assignment of 0 for ND samples) to 0.39 µg/kg (upper-bound estimate with assignment of LOD or LOQ for ND samples). The Committee decided to consider both the mean consumption of cereals from the five Global Environment Monitoring System (GEMS) regional diets (230 g in Europe) and the mean consumption of cereals for Germany from the 13 GEMS Food Contamination Monitoring and Assessment Programme (GEMS/Food) Consumption Cluster Diets (365 g, cluster E). The resulting dietary exposure estimate ranged from 8 to 17 ng/kg bw per week, which can be compared with the previous estimate of 25 ng/kg bw per week.

9. COMMENTS

9.1 Absorption, distribution, metabolism and excretion

Ochratoxin A is efficiently absorbed from the gastrointestinal tract, mainly in the small intestine. Information from a number of species shows that it is distributed via the blood mainly to the kidneys, with lower concentrations found in liver, muscle and fat. Specific transporters may be involved in the cellular uptake of ochratoxin A into the kidney, where it accumulates. Transfer to milk has been demonstrated in rats, rabbits and humans, but little ochratoxin A is transferred to the milk of ruminants, owing to efficient hydrolysis of the amide bond by microflora in the rumen, to yield phenylalanine and ochratoxin *alpha*. Ochratoxin *alpha*, a chlorinated dihydroisocoumarin, is the major metabolite of ochratoxin A in all species examined. This and minor hydroxylated metabolites of ochratoxin A that have been identified are all reported to be less toxic than ochratoxin A itself. Ochratoxin A is excreted in urine and faeces, and the relative contribution of each of these routes in different species is influenced by the extent of enterohepatic recirculation of ochratoxin A

and its binding to serum proteins. These factors are also important in the determination of the serum half-life of ochratoxin A, which varies widely among species. Ochratoxin A has a long half-life in some non-ruminant mammals, ranging from 1–1.5 days in mice, 2–5 days in rats and 3–5 days in pigs up to around 20 days in macaque and vervet monkeys and 35 days in a human volunteer.

9.2 Toxicological data

None of the new studies on nephrotoxicity, developmental toxicity, neurotoxicity or immunotoxicity that have appeared since the Committee's last evaluation would have an impact on the Committee's previous selection of minimal renal changes in the pig, observed at a dose of 8 µg/kg bw per day (the LOEL), as a critical effect for risk assessment.

In its previous evaluation, the Committee commented that the mechanism by which ochratoxin A causes renal tumours was unknown, noting that both genotoxic and non-genotoxic (epigenetic) modes of action had been proposed. Investigation of the mode of action of ochratoxin A in the kidney, with particular reference to carcinogenic effects, has been a key driver of much of the research conducted since then.

Several hypotheses on the mode of action of ochratoxin A as a carcinogen have been proposed, and evidence has been generated in support of each of them. Some of these would completely account for tumour formation, whereas others have been considered as possible contributors to tumour formation. They can be summarized as follows:

- genotoxicity from direct interaction of ochratoxin A or a reactive metabolite with DNA;
- generation of tumours secondary to chronic renal toxicity and compensatory cell proliferation;
- generation of tumours secondary to inhibition of phenylalanine–tRNA^{Phe} synthetase and protein synthesis;
- disruption of cell–cell signalling pathways and the process of cell division;
- alteration of intracellular calcium homeostasis;
- mitochondrial dysfunction leading to oxidative stress and indirect induction of DNA damage.

Concerning a genotoxic mode of action, divergent results have been obtained in the large number of genotoxicity assays on ochratoxin A, most of which were available at the time of the Committee's previous evaluation. In more recent comet assays, there was evidence of DNA damage in vitro and in vivo, including in rat kidney. However, these positive results were generally obtained with high ochratoxin A exposure levels and, where investigated, were indicative of oxidative damage. The Committee noted that whereas some investigators have previously reported formation of a number of different DNA adducts detectable by the ³²P-postlabelling technique under different in vitro and in vivo conditions, particularly following prolonged, high exposures to ochratoxin A, others have not been able to detect DNA adduct formation, despite, in some cases, using similar doses of

ochratoxin A and more sensitive techniques. The Committee concluded that a direct genotoxic mode of action, by demonstration of covalent binding to DNA with the formation of DNA adducts containing ochratoxin A or a metabolite of ochratoxin A, has not been confirmed.

Concerning non-genotoxic modes of action, a number of recent studies have addressed early changes associated with ochratoxin A exposure *in vitro* and *in vivo*, including indicators of oxidative stress, alterations in gene expression and cell signalling pathways, increased apoptosis, disruption of cell mitosis and increases in cell proliferation. Recent work has shown marked dose- and time-related increases in renal cell proliferation after 4 and 13 weeks of gavage administration of ochratoxin A to rats at the same doses that gave rise to renal tumour formation in the 2-year rat study (70 and 210 µg/kg bw per day, 5 days/week), with a NOAEL for cell proliferation at the same dose as that where no increase in tumour formation was observed in the 2-year rat study (21 µg/kg bw per day, 5 days/week). The Committee noted that cell proliferation, which is known to be effective in increasing tumour incidence via conversion of DNA damage into permanent mutations, may be a key event in the mechanism of tumour formation with ochratoxin A. Overall, the Committee considered that the evidence points to a number of non-genotoxic modes of action that could plausibly be involved in the generation of renal tumours, and this supports the previous decision to set a PTWI.

In order to provide additional information for the risk assessment, the Committee performed BMD modelling using the carcinogenicity data on ochratoxin A from the rat bioassay performed by the NTP in 1989 (National Toxicology Program, 1989). The combined adenoma and carcinoma data from male rat kidney, representing the most sensitive sex, species and target organ for ochratoxin A carcinogenicity, were used for modelling. Six different models were fitted to the dose–incidence data to estimate the lower limits of one-sided 95% confidence intervals on the BMD representing a 10% renal tumour incidence (BMDL_{10S}). The lowest BMDL₁₀ had a value of 15 µg/kg bw per day, 5 days/week, and the model showing the best fit had a value of 25 µg/kg bw per day, 5 days/week. Thus, for establishing the PTWI, the BMDL₁₀ does not provide a lower point of departure than the LOEL of 8 µg/kg bw per day for minimal renal toxicity changes in the pig.

9.3 Observations in humans

The earlier literature on the association between human exposure to ochratoxin A and the occurrence of Balkan endemic nephropathy and associated urinary tract tumours was summarized in the previous evaluation (Annex 1, reference 153). Contrary to the clear causal evidence of ochratoxin A–induced nephrotoxicity and kidney carcinogenicity in rodents, the significance of ochratoxin A for human health remains unclear from the available epidemiological evidence. Moreover, ochratoxin A exposure is only one of several hypotheses concerning an environmental etiology for Balkan endemic nephropathy.

Blood concentration of ochratoxin A appears to be a reliable biomarker of exposure in humans. In the Committee's previous evaluation (Annex 1, reference 153), the concentrations of ochratoxin A in blood samples from healthy persons,

obtained in surveys conducted in 17, mainly European, countries, ranged between 0.1 and 40 ng/ml (with an exceptional maximum of 160 ng/ml). The concentrations of ochratoxin A in blood samples obtained in more recent surveys from nine countries, four of which are European, ranged between 0.15 and 1.14 ng/ml, suggesting a possible decline in extreme peak values for blood concentrations compared with earlier surveys.

9.4 Analytical methods

Methods for analysing ochratoxin A were thoroughly reviewed in the previous report (Annex 1, reference 153). At that time, validated analytical methods were already available for the determination of ochratoxin A in maize, barley, rye, wheat, wheat bran, wholemeal wheat, roasted coffee, wine and beer. The best methods used LC following cleanup using immunoaffinity columns. Recent developments in analytical methodology are described in section 3 of this monograph.

9.5 Sampling protocols

The only recent development in this area has been the publication of extensive information on the parameters governing sample size for testing green coffee for ochratoxin A.

9.6 Fungi producing ochratoxin A

At the fifty-sixth meeting of JECFA (Annex 1, reference 152), it was reported that ochratoxin A is produced by three taxonomically distinct groups of fungi: a single *Penicillium* species, *P. verrucosum*; *Aspergillus ochraceus* and several related *Aspergillus* species; and *A. carbonarius*, with a small percentage of isolates of the closely related species *A. niger*. Since that report, some other *Aspergillus* and *Penicillium* species have been described as potential sources of ochratoxin A. Details of the revised taxonomy of the fungi producing ochratoxin A and of the physiology and ecology of these fungi are described in section 5.1 of this monograph.

9.7 Effects of processing

A few recent studies have examined the effect of processing on concentrations of ochratoxin A in wine, coffee and European cereals.

9.7.1 Wine

Vinification has consistently been reported to reduce ochratoxin A concentrations in wine, independent of the initial ochratoxin A concentration in grapes. Ochratoxin A is removed at each solid-liquid separation stage of the process.

9.7.2 Coffee

There is general agreement that roasting has an influence on ochratoxin A concentrations, but the percentage reduction reported has varied widely. Light

roasting causes reductions in ochratoxin A of 0–80%. Dark roasting (i.e. to a typical espresso coffee) may cause reductions of more than 90% in ochratoxin A content.

9.7.3 Milling and breadmaking

Using whole wheat contaminated with ochratoxin A, a combination of cleaning, scouring, removal of the bran fraction and baking caused an overall reduction of about 75% of ochratoxin A in white bread.

9.7.4 Extrusion

The extrusion of wholemeal wheat contaminated with ochratoxin A resulted in a reduction of no more than 40% of the toxin, even under the harshest conditions likely to be used in commercial practice.

9.8 Prevention and control

The principal fungi that produce ochratoxin A in foods—*Aspergillus carbonarius*, *A. westerdijkiae*, *A. steynii*, *Penicillium verrucosum* and *P. nordicum*—are not associated with plants and hence are not usually present in food crops before harvest. The control of ochratoxin A in foods, therefore, is basically a post-harvest problem. The basic concepts of good harvest practice, of drying crops rapidly and keeping them dry in storage, transport and processing systems, will ensure that crops remain essentially free of ochratoxin A. One exception is the entry of *A. carbonarius* into grapes before harvest.

9.8.1 Grapes, wine and other grape products

Once *A. carbonarius* has gained entry to a grape via damaged skin, the high sugar/high acid combination provides a perfect medium for ochratoxin A production. The keys to low ochratoxin A levels in wine are the reliance on prevention of infection of grapes by pathogens, rapid harvest if rain causes skin splitting, good harvest practice, including rejection of poor quality bunches, and a minimal delay between harvest and crushing. Control of ochratoxin A formation in dried vine fruits is less easy, because any pre-harvest infection with *A. carbonarius* will continue to develop during the early stages of drying.

9.8.2 Coffee

Aspergillus westerdijkiae, *A. steynii* and *A. carbonarius* are the major causes of ochratoxin production in green coffee. Infection of coffee cherries occurs during handling after harvest and in the drying yard. Many coffee-growing areas in the world are subjected to misty or rainy conditions after harvest, with consequent slow drying of the coffee cherries and ochratoxin A formation. However, good processing and drying regimes can prevent this problem.

9.8.3 Cereals

Where ochratoxin A occurs in cool temperate zone cereals, it is produced by the growth of *P. verrucosum*. Again, there is no evidence that this species infects

growing cereal plants or occurs in nature in grains before harvest. The key to controlling ochratoxin A in cereals is rapid drying; however, in cool temperate zones, grain is often harvested during moist or rainy conditions, and rapid drying may be difficult in practice.

There were limited data available to the Committee on cereals grown in tropical zones, but ochratoxin A has been found to occur in sorghum, maize and millet.

9.8.4 Meat products

If meat products are infected by *P. nordicum*, this occurs during the processing stages. Control of levels of fungal spores in the air will reduce this problem.

9.9 Dietary exposure assessment

9.9.1 Analysis of data submitted

Data on occurrence of ochratoxin A in cereals were submitted by Canada, Germany, Japan and the EU. In addition, data reporting the contamination of cereals in Nigeria, Ghana and Burkina Faso were submitted by FAO, and information regarding the contamination of cocoa and coffee beans was submitted by Côte d'Ivoire. [Table 8](#) in section 8.1 describes the various aspects of the distribution of ochratoxin A contamination in cereals. The most contaminated commodities in the German data set were rye and buckwheat, whereas the most contaminated commodities in the African data set were sorghum, maize and millets.

9.9.2 Estimation of the concentration of ochratoxin A in cereals

The critical effects of ochratoxin A relate to long-term exposure, and therefore the central tendency of the distribution of contamination should be used for dietary exposure assessments. Owing to the fact that generally more than 50% of analysed samples are below the LOD or LOQ, the use of the median value to represent this central tendency is problematic. Therefore, the international exposure assessments to date are based on the mean level of contamination.

The data from Africa were not used for the dietary exposure assessment because they were targeted samples with fungal contamination. Individual data were not available from either Canada or the EU. Finally, the Committee decided not to merge individual analytical data from Germany and Japan, but to focus the dietary exposure assessment on the largest data set of analytical results for raw and processed cereals from Germany.

9.9.3 Impact of left and right censorship on average level of contamination

Information on LOD and LOQ is essential for the purpose of the current assessment to assign values to ND results. The assignment of ND equal to 0 and ND equal to LOD or LOQ was used to provide a lower- and an upper-bound concentration of ochratoxin A in cereals.

Analytical results (detected or non-detected) for which information on LOD or LOQ was not available were removed from the data set. In addition, because available LOD or LOQ values were very variable, the impact of removing analytical data obtained with the highest LOQs was tested. The Committee excluded all samples (detected and non-detected) where the LOQ was higher than 1 µg/kg because the method of analysis was not very sensitive and there were sufficient data for the analysis obtained by more sensitive methods.

From the available data, it is possible to simulate the impact of various MLs, assuming 100% enforcement, on mean ochratoxin A concentration by excluding analytical results above each ML. Based on the available individual analytical results for raw cereals from Germany, the mean contamination was calculated using two scenarios for left censorship (i.e. ND = 0 and ND = LOD or LOQ), two scenarios for right censorship (i.e. ML at 5 or 20 µg/kg) and finally a scenario without an ML, resulting in six scenarios compiled in [Table 9](#) in section 8.2.2.

The available data show a limited impact of the various MLs on the mean contamination of raw cereals by ochratoxin A. However, it is important to note that the observed differences are due to very few high analytical results (15 samples out of 1462 excluded when the ML is 5 µg/kg and 6 samples out of 1462 excluded when the ML is 20 µg/kg).

9.9.4 Impact of new data on estimates of dietary exposure to ochratoxin A

In 2001, the mean overall dietary exposure to ochratoxin A, based mainly on European data, was estimated by the Committee to be 43 ng/kg bw per week (Annex 1, reference 152). The contribution from cereals was estimated to be 58% of the overall exposure (25 ng/kg bw per week), based on a daily consumption of 230 g of cereals and a mean ochratoxin A concentration of 0.94 µg/kg for raw cereals.

At the present meeting, mean concentrations of ochratoxin A for processed cereals were estimated to be 0.31 µg/kg for the lower bound and 0.39 µg/kg for the upper bound, based on German data ([Table 10](#) in section 8.2.2). In order to perform a realistic estimate of dietary exposure from the consumption of cereals, the Committee decided to consider both the mean consumption of cereals from the five GEMS regional diets (230 g in Europe) and the mean consumption of cereals from the 13 GEMS/Food Consumption Cluster Diets (365 g in cluster E, includes Germany). The resulting dietary exposure estimate ranged from 8 to 17 ng/kg bw per week, which can be compared with the previous estimate of 25 ng/kg bw per week.

10. EVALUATION

The new data, including data on mode of action of ochratoxin A in the kidney, do not indicate any reason to modify the previous approach taken by JECFA with respect to setting a PTWI. The Committee therefore retained the previous PTWI of 100 ng/kg bw.

The current estimate of overall dietary exposure to ochratoxin A from cereals, based mainly on European data, is about 8–17 ng/kg bw per week, based on processed cereals, compared with 25 ng/kg bw per week in the previous evaluation, based on raw cereals. The current estimate is well below the PTWI.

Contamination levels in the majority of raw cereal samples were below 5 µg/kg. Owing to the very small number of samples contaminated above the highest proposed limit of 20 µg/kg for cereals, such an ML would have very limited impact compared with no ML. The Committee concluded that the use of an ML of 5 or 20 µg/kg would be unlikely to have an impact on dietary exposure to ochratoxin A. The Committee was unable to reach a conclusion regarding the situation in developing countries, owing to the lack of adequate data with which to do an assessment.

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