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Alternaria mycotoxins: an overview of chemical characterization, producers, toxicity, analysis and occurrence in foodstuffs

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Abstract

Microfungi of the genus *Alternaria* are ubiquitous pathogens and saprophytes. Many species of the genus *Alternaria* commonly cause spoilage of various food crops in the field or post-harvest decay. Due to their growth even at low temperatures, they are also responsible for spoilage of these commodities during refrigerated transport and storage. Several *Alternaria* species are known producers of toxic secondary metabolites - *Alternaria* mycotoxins. *A. alternata* produces a number of mycotoxins, including alternariol, alternariol monomethyl ether, altenuene, altertoxins I, II, III, tenuazonic acid and other less toxic metabolites. Tenuazonic acid is toxic to several animal species, e.g. mice, chicken, dogs. Alternariol, alternariol monomethyl ether, altenuene and altertoxin I are not very acutely toxic. There are several reports on the mutagenicity and genotoxicity of alternariol, and alternariol monomethyl ether. Alternariol has been identified as a topoisomerase I and II poison which might contribute to the impairment of DNA integrity in human colon carcinoma cells. Analytical methods to determine *Alternaria* toxins are largely based on procedures, involving cleanup by solvent partitioning or solid phase extraction, followed by chromatographic separation techniques, in combination with ultraviolet, fluorescence, electrochemical and mass spectroscopic detection. A large number of *Alternaria* metabolites has been reported to occur naturally in food commodities (e.g. fruit, vegetables, cereals and oil plants). Alternariol, alternariol monomethyl ether and tenuazonic acid were frequently detected in apples, apple products, mandarins, olives, pepper, red pepper, tomatoes, tomato products, oilseed rape meal, sunflower seeds, sorghum, wheat and edible oils. Alternariol and alternariol monomethyl ether were detected in citrus fruit, Japanese pears, prune nectar, raspberries, red currant, carrots, barley and oats. Alternariol monomethyl ether and tenuazonic acid were detected in melon. Natural occurrence of alternariol has been reported in apple juice, cranberry juice, grape juice, prune nectar, raspberry juice, red wine and lentils.

Keywords: alternariol, alternariol monomethyl ether, altenuene, altertoxins, tenuazonic acid

1. Introduction

Several reviews on the *Alternaria* toxins have been published over the last few decades (Chełkowski and Visconti, 1992; Visconti and Sibilia, 1994; Panigrahi, 1997; Bottalico and Logrieco, 1998; Scott, 2001, 2004). *A. alternata* produces a number of mycotoxins, including alternariol, alternariol monomethyl ether, altertoxin I and tenuazonic acid, so it is a species of particular interest to mycotoxicologists. *A. alternata* f. sp. *lycopersici*, a rarely occurring pathotype of *A. alternata*, produces AAL-toxins. AAL-toxins are structurally related to fumonisins. There is only one report on their natural occurrence in hay silage (Yu *et al*., 1999). The toxicity of AAL-toxins to dairy cattle is not known but should be explored given the apparent abundance of these toxins. AAL-toxins are not presented in this review, because recent information about AAL-toxins is not available.

Alternaria toxins have recently received increasing attention, both in research programmes as well as in risk assessment studies. For example, a 4-year multidisciplinary project was conducted, initiated within the Fifth Research Framework Programme of the European Union. This project 'Safe organic vegetables and vegetable products by reducing risk factors and sources of fungal contaminants throughout the production chain: the carrot - *Alternaria* model' was carried out from 2000 to 2004. The objective of the project was to develop strategies for a safe organic food supply by developing detection methods, identifying *Alternaria* mycotoxin risks in the production chain, determining the critical control points, and developing preventive measures. The research was performed using the model system carrot – *Alternaria*. Carrot is one of the most popular organic vegetables bought by European consumers and is an important component of various organic food products, such as baby food and vegetable juices. Based on these findings and previous reports, it was concluded that *Alternaria* mycotoxins in carrots do not represent a hazard for consumers (Solfrizzo *et al.*, 2004, 2005). The German Federal Institute of Risk Assessment has recently pointed out that there is an urgent need for more information about the toxicity of *Alternaria* toxins, because data published so far do not allow assessment of the health risk for the consumer (BfR, 2003). A scientific opinion in 2007 of the Czech Scientific Committee on Food on *Alternaria* mycotoxins led to the same conclusion (CSCF, 2007).

The present review will focus on the most important *Alternaria* mycotoxins and their chemical characterisation, producers, toxicity, analytical methods and natural occurrence in foodstuffs and foods. Special emphasis will be given to hazard characterisation.

2. Chemical characterisation of *Alternaria* mycotoxins

More than 30 potentially toxic products have been isolated from *Alternaria* species. *Alternaria* species produce many secondary metabolites, mycotoxins and mostly host-specific or non-host specific phytotoxins, which play an important role in the pathogenesis of plants (Templeton *et al.*, 1967; Pero and Main, 1969; Tirokata *et al.*, 1969, Coombe *et al.*, 1970; Pero *et al.*, 1973a; Ueno *et al.*, 1975; Janardhanan and Husein, 1975; Maekawa *et al.*, 1984; Rizk *et al.*, 1985; Tadakazu *et al.*, 1985; Liebermann *et al.*, 1988; Visconti *et al.*, 1988; Logrieco *et al.*, 1990; Thomma, 2003). Reviews on *Alternaria* toxins and their structures are found in Templeton (1972), Harvan and Pero (1976), Schade and King (1984), Chełkowski and Visconti (1992), and Bottalico and Logrieco (1998). Several reviews on the *Alternaria* toxins have been more recently published and are listed by Scott (2001).

The most important *Alternaria* mycotoxins are alternariol (AOH), alternariol monomethyl ether (AME), altenuene (ALT), altertoxins I, II, III (ATX-I, -II, -III) and tenuazonic acid (TeA), which belong to three structural classes (Bottalico and Logrieco, 1998):

- dibenzopyrone derivatives (AOH, AME, ALT)
- perylene derivatives (ATX-I, -II, -III)
- tetramic acid derivatives (TeA).

The chemical structures of these mycotoxins as outlined below, are given in Figure 1 (CTD, 2008):

AOH: (3,7,9-trihydroxy-1-methyl-6*H*-dibenzo[*b,d*]pyran-6-one); CAS (Chemical Abstracts Services) Registry No. 641-38-3; M.W. (molecular weight) 258; molecular formula $C_{14}H_{10}O_5$.

AME: 3,7-dihydroxy-9-methoxy-1-methyl-6*H*-dibenzo-[*b,d*]pyran-6-one; CAS Registry No. 26894-49-5 (or 23452-05-3); M.W. 272; molecular formula $C_{15}H_{12}O_5$.

ALT: (2α,3α,4aβ-tetrahydro-2,3,7-trihydroxy-9-methoxy-4a-methyl-6*H*-dibenzo[*b,d*]pyran-6-one); CAS Registry No. 29752-43-0; M.W. 292; molecular formula $C_{15}H_{16}O_6$.

ATX-I: 1,2,7,8,12b-pentahydro-1,4,6b,10-tetrahydroxy-perylene-3,9-dione; CAS Registry No. 56258-32-3; M.W. 352; molecular formula $C_{20}H_{16}O_6$.

ATX-II: [perylo(1,2-b)oxirene-7,11-dione,7a,8a,8b,8c,9,10-hexahydro-1,6,8c-trihydroxy-, (7aR,8aR,8bS,8cR)-]; CAS Registry No. 56257-59-1; M.W. 350; molecular formula $C_{20}H_{14}0_6$.

ATX-III: [perylo(1,2-b:7,8-b')bisoxirene-5,10-dione, 1a,1b,5a,6a,6b,10a-hexahydro-4,9-dihydroxy-]; CAS Registry No. 105579-74-6; M.W. 348; molecular formula $C_{20}H_{12}0_6$.

TeA: (3-acetyl-5-*sec*-butyl-4-hydroxy-3-pyrrolin-2-one); CAS Registry No. 610-88-8; M.W. 197; molecular formula $C_{10}H_{15}O_3N$.

The most important *Alternaria* mycotoxins were first isolated, characterised and confirmed in the years 1953-1986. A chronological survey is shown in Table 1. To provide sufficient amounts of material for toxicological and biological testing, total syntheses of AOH, AME, ALT and iso-ALT were established (Koch *et al.,* 2005; Altemöller *et al.*, 2006). The possible formation of conjugated *Alternaria* mycotoxins, in which the mycotoxin is usually bound to a more polar substance like glucose analogous to ZEA-4-glucoside and DON-3-glucoside (Gareis *et al.*, 1990; Berthiller *et al.*, 2005), was discussed by P.M. Scott and V. Ostry (personal communications). It was established that the occurrence of AOH and AME glycoside conjugates in foodstuffs is possible.

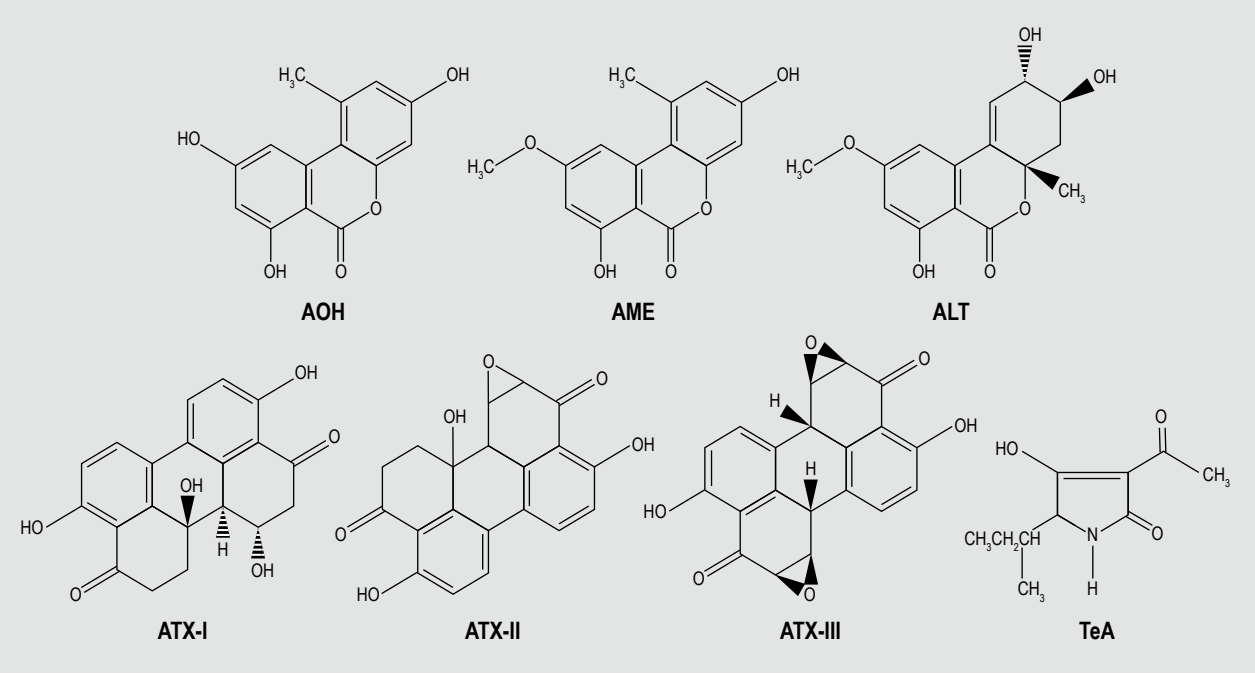

Figure 1. Chemical structure of AOH, AME, ALT, ATX-I, ATX-II, ATX-III and TeA.

Table 1. Chronological survey of the isolation of the most important *Alternaria* mycotoxins.

Mycotoxin	Year of isolation	Reference
AOH	1953	Raistrick *et al.*, 1953; Thomas, 1961; Pero and Main, 1969; Tirokata *et al.*, 1969
AME	1953	Raistrick *et al.*, 1953; Thomas, 1961; Pero and Main, 1969; Tirokata *et al.*, 1969
TeA	1957	Rosset *et al.*, 1957; Janardhanan and Husein, 1975
ALT	1971	Pero *et al.*, 1971; Rizk *et al.*, 1985
ATX-I, -II, -III	1973	Pero *et al.*, 1973b; Chu, 1981; Stack *et al.*, 1986; Stack and Prival, 1986

3. Producers of *Alternaria* mycotoxins

Alternaria mycotoxins are naturally occurring secondary metabolites produced by toxigenic microfungi of *Alternaria* species, which develop on agricultural crops (King and Schade, 1984; Chełkowski and Visconti, 1992; Bottalico and Logrieco, 1998; Patriarca *et al.*, 2007). *Alternaria* species infest numerous foodstuffs but also grow on other materials, e.g. soil, wall papers and textiles. Decaying wood, wood pulp and compost are likewise favourite substrates for this species (Gravesen *et al.*, 1994). The genus *Alternaria* was originally described in 1816 (Nees von Esenbeck, 1816). Many species of *Alternaria* have been described since then. The Index fungorum shows 491 records of *Alternaria* species (CABI, 2004).

A. alternata (Fr.) Keissler (= *A. tenuis* Nees) is the most abundant of more than forty *Alternaria* species. *A. tenuis* was originally described in 1816 as the type and only member of the genus *Alternaria* (Nees, 1816). In his monumental work, Systema Mycologicum, Fries (1832) did not recognise Nees von Esenbeck´s description of *A. tenuis*, and cited Nees' species as a synonym of *Torula alternata*. Von Keissler (1912) synonymised both *A. tenuis* and *T. alternata* with *A. alternata* Keissl. nov. nom. *A. alternata* (Fr.) Keissler (teleomorph = *Lewia* M.E. Barr & E.G. Simmons) is the correct name. Further changes appear unlikely (Simmons, 1986, 1992, 1995; Pitt and Hocking, 1997). However, recent taxonomic studies suggest that *A. alternata* may include more than one species and taxonomic revision can be expected (Pitt and Hocking, 1997). The Index fungorum shows 4 records of *A. alternata* (CABI, 2004).

Optimum growth of *A. alternata* is near 25 °C with minima variously reported as -5 to 6.5 °C and maxima near 36 °C (Hasija, 1970; Domsch *et al.*, 1980; Pitt and Hocking, 1997). Gravesen *et al.* (1994) reported that temperature requirements for growth are minimum 2 °C, maximum 32 °C, and the optimum temperature lies between 25 and 28 °C. The minimum a_w for growth at 25 °C is 0.88 (Hocking *et al.*, 1994). Optimal growth occurs at pH 4-5.4,

and the pH range for growth is 2.7-8.0 (Hasija, 1970). *A. alternata* is able to grow in oxygen concentrations as low as 0.25% (v/v) in N_2, with growth rates being proportional to oxygen concentration (Follstad, 1966; Wells and Uota, 1970). Maximum production of AOH, AME and ALT occurs at 25 °C and 0.98 a_w (Magan *et al*., 1984). However, the optimum for TeA production was reported at 25 °C and 0.90 a_w (Young *et al.*, 1980; Etcheverry *et al.*, 1994).

A. alternata produces important mycotoxins: AOH, AME, ALT, iso-ALT, TeA and ATX-I, -II, -III (King and Schade, 1984; Visconti *et al.*, 1986; Logrieco *et al.*, 1990; Ozcelik *et al*., 1990; Ansari and Shrivastava, 1990; Sanchis *et al*., 1993; Müller, 1992; Bilgrami *et al.*, 1994; Andersen and Thrane, 1996). The production of important *Alternaria* mycotoxins by *Alternaria* species other than *A. alternata* can be seen in Table 2.

TeA is also produced by other species of fungi including, *Pyricularia oryzae* and *Phoma sorghina* (Iwasaki *et al.*, 1972; Steyn and Rabie, 1976; Bottalico and Logrieco, 1998).

4. Toxicity of *Alternaria* toxins

Exposure to *Alternaria* toxins has been linked to a variety of adverse health effects. Several reviews on toxicology of the *Alternaria* toxins have been published and are listed by Harvan and Pero (1976), Chełkowski and Visconti (1992), Woody and Chu (1992), Liu *et al*. (1992), Visconti and Sibilia (1994), Panigrahi (1997), and Bottalico and Logrieco (1998). *Alternaria* isolates grown in laboratory culture are toxic to chickens and rats (Sauer *et al*., 1978), chicken embryos (Griffin and Chu, 1983) and human cell cultures (Pero *et al.*, 1973b). *Alternaria* laboratory cultures are also teratogenic and foetotoxic in mice (Pero *et al.*, 1973b; Harvan and Pero 1976; Griffin and Chu 1983). Crude culture extracts of *Alternaria* spp. are mutagenic in the Ames test (Scott and Stoltz, 1980; Schrader *et al*., 2001). Extracts of *Alternaria alternata* induced reverse mutation in *Escherichia coli*, unscheduled DNA synthesis in cultured human amnion FL cells, chromosomal aberrations and sister chromatid exchange in human peripheral blood lymphocytes, mutation in V79 cells and transformation of NIH3T3 cells (Liu *et al.,* 1991). A maize flour culture of *A. alternata* was carcinogenic to rats and other culture extracts were mutagenic in various *in vitro* systems. Recently, liver and kidney damage in rats was produced by feeding *A. alternata* for 28 days (Combina *et al.,* 1999).

The possibility that *A. alternata* may be a factor in the aetiology of oesophageal cancer in Linxian, China has been suggested by Dong *et al.* (1987), Zhen *et al.* (1991) and Liu *et al.* (1991, 1992). The mutagenicity and carcinogenicity of AME, AOH, and their relevance to the etiology of human oesophageal cancer were studied. These mycotoxins were produced by *A. alternata*, which was the main contaminating fungus isolated from the grain in Linxian County, an area with high incidence of oesophageal cancer. This study demonstrated that: (1) AME and AOH might cause cell mutagenicity and transformation, (2) AME and AOH could combine with the DNA isolated from human foetal oesophageal epithelium, activate the oncogens, c-H-ras and c-mys in it, and promote proliferation of human foetal oesophageal epithelium in vitro, (3) squamous cell carcinoma of the foetal oesophagus could be induced by AOH. According to these results, (Liu *et al.*, 1992) conclude

Table 2. The production of important mycotoxins by *Alternaria* species (excluding *A. alternata*).

Species	Mycotoxin	Reference
A. brassicae (Berk.) Sacc.	AOH, AME	Bottalico and Logrieco, 1998
A. capsici-anui Săvul. & Sandu	AOH, AME, TeA	Bottalico and Logrieco, 1998
A. cassiae Jurair & A. Khan	ATX-I, -II	Hradil *et al.*, 1989
A. citri Ell. & Pierce	AOH, AME, TeA	Freeman, 1965; Kinoshita *et al.*,1972
A. cucumerina (Ell. & Ev.) Elliott	AOH, AME	Raistrick *et al.*,1953; Freeman, 1965
A. dauci (Kühn) Groves & Skolko	AOH, AME	Freeman, 1965; Raistrick *et al.*, 1953
A. japonica Yoshii	TeA	Kinoshita *et al.*, 1972
A. kikuchiana Tanaka	AOH, AME, TeA	Tirokata *et al.*, 1969; Kinoshita *et al.*, 1972; Kameda *et al.*, 1973
A. longipes (Ell. & Ev.)	AME, TeA	Mikami *et al.*, 1971; Bottalico and Logrieco, 1998
A. mali Roberts	ATX-I, -II, -III, TeA	Kinoshita *et al.*, 1972
A. oryzae Hara	TeA	Kinoshita *et al.*, 1972
A. porri (Ell.) Cif.	AME, TeA	Bottalico and Logrieco, 1998
A. radicina Meier, Drechsler & Eddy	ATX-I, -II, -III, TeA	Bottalico and Logrieco, 1998; Solfrizzo *et al.*, 2005
A. solani Sorauer	AOH, AME, TeA	Stoessl, 1969; Pollock *et al.*,1982; Bottalico and Logrieco, 1998
A. tenuissima (Kunze) Wiltshire	AOH, AME, ATX-I, -III, TeA	Davies *et al.*, 1977; Young *et al*., 1980; Bottalico and Logrieco, 1998
A. tomato (Cooke) Jones	AOH, AME, ATX-I, -II, -III, TeA	Bottalico and Logrieco, 1998

that *A. alternata* plays an important role in the etiology of human oesophageal cancer.

The toxicities of several *Alternaria* mycotoxins to brine shrimp (*Artemia salina* L.) larvae were determined using the disc method of inoculation with an 18-hour exposure period. The 50% lethal concentration dose of TeA, AOH, ATX-I and ALT were, respectively, around 75, 100, 200 and 375 g/ml. AME was found not to produce any mortality, however, assessment of its toxicity was unreliable because its poor solubility in the common solvents posed testing difficulties (Panigrahi and Dallin, 1994).

Hazard identification

Alternariol and related metabolites

AOH, AME and ALT are not very acutely toxic (Pero *et al.*, 1973b; Pollock *et al.*, 1982; Olsen and Visconti, 1987). There are several reports on the mutagenicity and genotoxicity of AOH and AME (Scott and Stoltz, 1980; McCann *et al.*, 1981; DiCosmo and Straus, 1985; Yu-Hui *et al.*, 1986; An *et al.*, 1989; Davis and Stack, 1994; Schrader *et al.*, 2001, 2006; Brugger *et al.*, 2006). In the study by Brugger *et al.* (2006) the mutagenicity of AOH is investigated at the hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene locus in V79 cells and at the thymidine kinase (TK) gene locus in mouse lymphoma L5178Y $tk^{+/-}$ cells (MLC). Concentrations of 10 µM AOH and more gave rise to a significant and concentration-dependent induction of HPRT and TK mutations in V79 cells and in MLC, respectively. The mutagenic potency of AOH was about 50-fold lower than that of the established mutagen 4-nitroquinoline-*N*-oxide in both cell lines. Discrimination between small and large colonies in the TK assay revealed the predominant induction of small colonies, which are indicative for extensive chromosomal deletions and which correlated with the induction of micronuclei in MLC. The mutagenicity of AOH may have a bearing on the carcinogenicity of this mycotoxin. However, various inconsistencies are evident when the results of these studies are compared. For example, although AME and AOH are strongly mutagenic in both the *Bacillus subtilis* rec assay, a repair test which compares zones of inhibition induced by a test chemical on lawns of wild type and rec– bacteria (Kada *et al.*, 1984) and *E. coli* ND160 reverse mutation assay (Yu-Hui *et al.*, 1986; An *et al.*, 1989; Zhen *et al.*, 1991), which measures frameshift revertants at a lacZ mutation (Clarke and Wade, 1975), the compounds are non- to weakly mutagenic in the Ames *Salmonella* test using *S. typhimurium* strains TA98 and TA100 (Scott and Stoltz, 1980, Davis and Stack, 1994, Schrader *et al.*, 2001). In addition, the low or questionable mutagenic activities found for several of the individual toxins seem unable to account for the perceived carcinogenic threat, suggesting that an additional mechanism could be involved in mutagenic activation (Schrader *et al.*, 2006).

In most assays, AOH appears to exhibit a more pronounced genotoxicity than AME. Marko (2007) investigated the genotoxic effect of the *Alternaria* toxins: AOH, AME, ALT and iso-ALT with emphasis on potential mechanisms involved. AOH and AME significantly increased the rate of DNA strand breaks in human colon carcinoma cells at concentrations ≥1 µM and 25 µM, respectively, measured as DNA strand breaks by single cell gel electrophoresis (comet assay). In contrast, ALT and iso-ALT did not affect DNA integrity up to 100 µM. The rate of DNA strand breaks induced by AOH and AME were not modulated by formamidopyrimidine-DNAglycosylase (FPG), thus excluding enhanced oxidative DNA damage. AOH effectively competed with the minor groove binding ligand Hoechst 33258 with an EC_{50}-value of 8±1 µM indicating substantial affinity to the minor groove of the DNA.

A number of DNA minor groove binding ligands affect mammalian topoisomerases I and II. Therefore, Marko (2007) investigated the impact of *Alternaria* toxins on the different classes of topoisomerases. AOH was found to inhibit the catalytic activity of topoisomerase I at concentrations ≥50 µM. In contrast AME, bearing a methoxy group at position 9, did not affect the catalytic activity of topoisomerase I up to 100 µM. The catalytic activity of topoisomerase IIα and IIβ was significantly suppressed by AOH at ≥25µM and ≥150 µM, respectively. AME did not affect the activity of topoisomerase IIβ up to 200 µM, but was found to be equipotent to AOH with respect to the inhibition of topoisomerase IIα. Thus, topoisomerase IIα was identified as the most sensitive target so far for both AOH and AME. In accordance with the results in the comet assay, ALT and iso-ALT did not affect the activity of topoisomerase I and II.

Furthermore, Marko (2007) investigated the mode of interaction of AOH with the potential target enzymes. AOH was found to stabilise the catalytically generated DNA-topoisomerase intermediate of topoisomerase I and II, thus acting as a so-called topoisomerase poison. The stabilisation of the DNA-topoisomerase IIα intermediate was observed in the concentration range leading to enhanced DNA strand breaks in the comet assay. In summary, AOH and AME were identified as potent inhibitors of topoisomerase IIα, which might at least contribute to the DNA strand breaking properties of these mycotoxins. In a study by Pfeiffer *et al.* (2007), the DNA strand-breaking activity of the two *Alternaria* mycotoxins has been compared in three cell lines with different metabolic capabilities, i.e. Chinese hamster V79 and human HepG2 and HT-29 from liver and intestine, respectively. DNA single strand breaks induced by the test compounds were determined by using the technique of alkaline unwinding. AOH und AME were found to induce

strand breaks in a concentration-dependent manner in all three cell lines. The incidence was about equal for AOH and AME in V79 cells which do not metabolise the mycotoxins, and exceeded the incidence observed in HepG2 and HT-29 cells. The human cells were not capable of oxidative metabolism but exhibited UDP-glucuronosyl transferase (UGT) activity. AOH was more extensively glucuronidated than AME in HepG2 cells, resulting in a higher incidence of strand breaks upon treatment of these cells with AME. HT-29 cells were much more efficient than HepG2 cells in glucuronidating AOH and AME. For example, 24 h after incubating HepG2 cells to AOH or AME, about 50 % of the dose was still present as unconjugated compound, but the rate of strand breaks was similar to an incubation of 1 h. In contrast, AOH and AME were completely glucuronidated in HT-29 cells after 24 h, and strand breaks were no longer detectable at this time point. Our results suggest that glucuronidation of AOH and AME suppresses the genotoxic activity of these mycotoxins. In support of this assumption is the observation that curcumin, a known inhibitor of UGT activity, increased the incidence of AOH- and AME-induced strand breaks in HT-29 cells.

AOH has been reported to possess cytotoxic, genotoxic and mutagenic properties *in vitro.* Fehr *et al*. (2007) investigated the genotoxic effect of AOH in human colon carcinoma cells with special emphasis on the potential mechanisms of action. Interference with human topoisomerases can cause profound DNA damage. During the catalytic cycle topoisomerases introduce transient breaks in the phosphodiester backbone of the DNA, enabling the release of torsion stress and thereby regulating the topology of the DNA during all essential processes of DNA metabolism, such as transcription, replication, chromosome condensation and segregation. In mammals two major classes of topoisomerases exist, classified due to their mode of action. Topoisomerase I introduces a transient single strand break in the DNA double helix. In contrast, topoisomerase II, an ATP-dependent enzyme, performs a transient double strand break, through which a second DNA double helix is passed. During these processes a transient covalent enzyme-DNA-intermediate is formed, the so-called cleavable complex. The majority of compounds targeting topoisomerases affect the stability of the cleavable complex. As a consequence the collision with an approaching replication fork might lead to fatal DNA strand breaks. Fehr *et al*. (2007) investigated the DNA strand breaking potential of AOH in human colon carcinoma cells (HT29) by single cell gel electrophoresis. Within one hour of incubation, AOH significantly increased the rate of DNA strand breaks at concentrations ≥1 μM indicating substantial genotoxic potential. In a cell-free system, AOH was found to inhibit the catalytic activity of topoisomerase I at concentrations ≥50 μM. The catalytic activity of topoisomerase IIα and IIβ was significantly suppressed by AOH at ≥25 μM and ≥150 μM, respectively, indicating a preference of this mycotoxin towards the IIα isoform. Furthermore, the question was addressed whether AOH acts as a pure catalytic inhibitor or as a topoisomerase poison, stabilising the cleavable complex. In HT29 cells, the inhibition of topoisomerase I and II activity was associated with the stabilisation of the topoisomerase I- and II-DNA-complexes thus identifying AOH as a topoisomerase I and II poison. In summary, AOH was identified as a topoisomerase I and II poison which might cause or at least contribute to the impairment of DNA integrity in human colon carcinoma cells.

There is also some evidence of carcinogenic properties of AOH and AME: squamous cell carcinoma were induced in mice subcutaneously inoculated with human embryo oesophageal tissue that had been treated with AOH; and NIH/3T3 cells transformed by AME were subcutaneously tumorigenic in mice. No cancer studies of these *Alternaria* mycotoxins in animals have been carried out. However, precancerous changes were recently observed in oesophageal mucosa of mice fed 50-100 mg/kg body weight (b.w.) per day of AME for 10 months (Yekeler *et al.*, 2001).

Lehmann *et al.* (2006) investigated the estrogenic potential, the effect on cell proliferation, and the genotoxic effect of AOH in cultured mammalian cells. AOH replaced E2 from isolated human oestrogen receptors α and β and increased the level of alkaline phosphatase (ALP) mRNA and the enzymatic activity of ALP in a human endometrial adenocarcinoma cell line (Ishikawa cells). The oestrogenicity of AOH was about 0.01% of that of E2. The effects in Ishikawa cells were reversed by the ER antagonist ICI 182,780. Analysis of cell proliferation by flow cytometry and microscopy of Ishikawa and Chinese hamster V79 cells revealed that AOH inhibited cell proliferation by interference with the cell cycle. The genotoxic potential was assessed by the micronucleus (MN) assay and immunochemical differentiation between MN containing whole chromosomes (kinetochore-positive) and DNA fragments (kinetochore-negative) in Ishikawa and V79 cells. AOH induced kinetochore-negative MN in both cell lines. This is the first report on the estrogenic potential, inhibition of cell proliferation and clastogenicity of AOH in Ishikawa and V79 cells *in vitro*.

Altertoxins-I, -II, -III

ATXs are mutagenic in the Ames test. Ames *Salmonella* strains TA98 and TA100 were used (Scott and Stoltz, 1980). The mutagenic activity of ATXs was confirmed by Stack *et al.* (1986) and Stack and Prival (1986). ATX-I and the related perylene derivatives ATX-II and -III are more potent mutagens and acute toxins to mice than AOH and AME (Woody and Chu, 1992; Chełkowski and Visconti, 1992; Schrader *et al.*, 2001). In the study by Schrader *et al*. (2006) ATX-I was re-examined ± nitrosylation, using

Ames *Salmonella* strain TA97, as well as strains TA102 and TA104 that are more sensitive to oxidative damage. ATX-I was also assessed for mammalian mutagenicity at the Hprt gene locus in Chinese hamster V79 lung fibroblasts and rat hepatoma H4IIE cells. When tested from 1 to 100 µg/plate without nitrosylation, ATX-I was mutagenic in TA102 ± rat liver S9 for activation and weakly mutagenic in TA104 ± S9, demonstrating direct-acting AT base pair mutagenicity. Nitrosylation of ATX-I enhanced mutagenicity at AT sites in TA104 ± S9 but produced little change in TA102 ± S9 compared to native ATX-I. However, nitrosylated ATX-I generated a potent direct-acting frameshift mutagen in TA97 ± S9. While ATX-I was not mutagenic in either V79 cells or H4IIE cells, 5 and 10 µg/ml nitrosylated ATX-I produced a doubling of 6-thioguanine resistant V79 colonies and 0.5 and 1 µg/ml were mutagenic to H4IIE cells, becoming toxic at higher concentrations. Nitrosylated ATX-I was also directly mutagenic in mammalian test systems.

ATX I and III tested positively in a cell bioassay that detects tumor promoters. ATX-I and ATX-III were examined for activity in the Raji cell Epstein-Barr virus early antigen (EBV-EA) induction system and in the C3H/10T murine fibroblast cell transformation system. Exposure of Raji cells to ATX-I or ATX-III activated EBV-EA expression by 8- and 9.5-fold, respectively. A single exposure of C3H/10T cultures to ATX-I or ATX-III resulted in significant increases in cell transformation, and the response to ATX-I was stronger. Both ATXs enhanced the transformation of C3H/10T cells, and chronic exposure of non-initiated C3H/10T cells to ATX-I and ATX-III, starting 6 days after cells were plated, resulted in cell transformation in 8/59 and 12/37 dishes, respectively, compared with transformation in only 2/63 control dishes. Since activation of EBV-EA in Raji cells has been positively correlated with tumour promoters, these data together indicate that ATX-I and ATX-III have a potential role in cell transformation (Osborne *et al.*, 1988).

Tenuazonic acid

TeA is toxic to several animal species, e.g. mice, chicken, dogs. In dogs, it caused haemorrhages in several organs at daily doses of 10 mg/kg b.w. and in chickens sub-acute toxicity was observed with 10 mg/kg in the feed. In particular, increasing TeA in chicken feed from sublethal to lethal levels progressively reduced feed efficiency, suppressed weight gain and increased internal haemorrhaging. TeA is more toxic than AOH, AME and ALT (Steyn and Rabie, 1976; Griffin and Chu, 1983; Woody and Chu, 1992; Bottalico and Logriego, 1998; Logrieco *et al.*, 2003).

TeA is not mutagenic in bacterial systems (Scott and Stoltz, 1980; Schrader *et al.*, 2001). Precancerous changes were observed in oesophageal mucosa of mice fed 25 mg/kg b.w. per day of TeA for 10 months (Yekeler *et al.*, 2001). Sorghum grain colonised by *Phoma sorghina* that contained TeA was associated with the human haematological disorder known as 'Onyalai' (Steyn and Rabie, 1976; Bottalico and Logriego, 1998).

Hazard characterisation

The toxicity data of the *Alternaria* mycotoxins (AOH, AME, ALT and TeA) relevant for hazard characterisation are not available. No-observed-adverse-effect-levels (NOAEL), lowest-observed-adverse-effect-levels (LOAEL) and benchmark doses (BMD) could not be established for *Alternaria* mycotoxins (AOH, AME, ALT, ATX-I-II) for different endpoints in the studies described above. Exposure limits as tolerable daily intake (TDI) or a provisional maximum tolerable daily intake (PMTDI) in the terminology of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) for *Alternaria* toxins have not been derived by JECFA and the European Food Safety Authority (EFSA).

5. Analytical methods for *Alternaria* mycotoxins

Analytical methods for *Alternaria* mycotoxins were reviewed in 1984 by Schade and King and were last reviewed in 2001 by Scott. *Alternaria* mycotoxins are usually extracted from solid foods with organic solvents such as dichloromethane, methanol, acetonitrile, or ethyl acetate, while for TeA it is preferable to have an acidic extraction solvent (as cited by Scott 2001). Cleanup procedures of analytical methods for methods to determine *Alternaria* mycotoxins in foodstuffs include solvent partitioning (e.g. 5% $NaHCO_3$ followed by acidification and back-extraction into methylene chloride), generally used for TeA, and solid-phase extraction (SPE) columns (e.g. C_{18}, aminopropyl, SPE /Abselut - Nexus bonded phase, SPE /Oasis®) for AOH, AME, and ATX-I (Scott 2001). Solid phase microextraction (SPME) for TeA was also applied (Aresta *et al.*, 2003).

Alternaria mycotoxins have been determined after separation by thin-layer chromatography (TLC), high performance thin-layer chromatography (HPTLC), gas chromatography (GC), and more usually liquid chromatography (LC), mainly with ultraviolet (UV) detection, although fluorescence and electrochemical detection have also been used for *Alternaria* mycotoxins other than TeA. A Zn^{2+} salt is usually added to the LC mobile phase for TeA, a toxin of the tetramic acid class. Recently, atmospheric pressure chemical ionisation (APCI) LC-mass spectroscopy (MS) and LC-MS/MS have been applied to the determination and confirmation of identity of AOH and AME, e.g. in apple juice and other fruit beverages at sub ng/ml levels (Scott, 2001; Scussel *et al.*, 2007). A multi-method was developed with which 33 mycotoxins (including AOH and AME) in various products (peanut, pistachio, wheat, maize, cornflakes, raisins and figs) could be analysed simultaneously. The mycotoxins were

extracted with an acetonitrile/water mixture, diluted with water and then directly injected into a LC-MS/MS system. The mycotoxins were separated by reversed-phase LC and detected using an electrospray ionisation interface (ESI) and MS/MS, using multiple reaction monitoring (MRM) in the positive ion mode to increase specificity for quality control (Spanjer *et al*., 2008).

In the study by Lau *et al.* (2003) for determination of AOH and AME by LC-MS/MS, apple juice and other fruit beverages were cleaned up on C_{18} and aminopropyl solid-phase extraction columns. Positive and negative ion mass spectra of AOH and AME under ESI and APCI conditions were obtained. Collision-induced dissociation of the $[M+]^+$ and $[M-H]^-$ ions for both compounds were also studied. The phenolic anions of both compounds are more stable with less fragmentation. In quantitative analysis, negative ion detection also offers lower background and better detectability. Sensitive LC-MS and LC-MS/MS confirmatory procedures based on APCI with negative ion detection were applied to confirm the natural occurrence of AOH in nine samples of apple juice and in single samples of some other clear fruit beverages – grape juice, cranberry nectar, raspberry juice, red wine, and prune nectar (which also contained 1.4 ng AME/ml) – at levels of up to 6 ng AOH/ml. Electrospray LC-MS/MS with negative ion detection and in multiple reaction monitoring mode offers better detectability and specificity. Absolute detection was better than 4 pg per injection for both compounds.

In a study by Solfrizzo *et al.* (2004) an LC method was developed for the determination of *A. radicina* and *A. alternata* toxins in carrots. Toxins were extracted from carrot with an acidified mixture of water-methanol-acetonitrile. The filtered extract was divided in 2 parts that were purified by solid-phase extraction on a C_{18} column for the analysis of radicinin (RAD), ATX-I, AOH, and AME, and on a polymeric Oasis® HLB column for TeA. Toxins were quantified by reversed-phase LC with UV diode array detection by using 2 consecutive isocratic mixtures of acetonitrile–sodium dihydrogen phosphate solution. The limit of detection (LOD) (signal-to-noise ratio of 3) for RAD, TeA, ATX-I, AME, and AOH was 0.006, 0.02, 0.02, 0.01, and 0.005 µg/g, respectively.

The *Alternaria* mycotoxins AOH, AME have been determined by LC-MS/MS in white and red wines, grape juices and cranberry juices. After clean-up on aminopropyl SPE columns AOH and AME were initially determined by reversed phase LC with UV detection. Positive sample extracts were re-analysed by LC-tandem negative ion electrospray mass spectrometry in multiple reaction monitoring mode. Overall mean method recoveries measured by LC-UV were 93% for AOH and 81% for AME. LOD (signal-to-noise ratio of 3) in wine (and juice) by LC-UV for AOH were 0.8 (0.4) ng/ml and for AME were 0.5 (0.4) ng/ml; they were below 0.01 ng/ml by LC-MS/MS (Scott *et al.*, 2006).

Extract clean-up by partitioning (two clean-up procedures) has been described. The final separation and quantification of seven *Alternaria* toxins (AOH, AME, ALT, ATX-I, TeA, tentoxin, and the AAL-toxins TA-1 and TA-2) from edible oil and oilseeds has employed LC-MS/MS (Kocher, 2006, 2007). LOD (signal-to-noise ratio of 3) for AOH, AME, ALT, ATX-I, TeA, tentoxin, and TA-1 and TA-2 was 0.07, 0.05, 0.25, 0.05, 0.86, 0.11, 0.14 and 0.05 µg/kg, respectively.

A simple and accurate planar chromatography method (instrumental HPTLC with densitometric determination) of AOH, AME, ALT and TeA in wheat, rapeseeds, lentils and wine was reported by Ostry *et al.* (2004), Skarkova *et al.* (2005) and Ostry *et al.* (2007). Clean-up procedures of analytical methods for foodstuffs include solvent partition (e.g. methanol/5% $(NH_4)_2SO_4$, defatting by n-hexane, back-extraction into methylene chloride or chloroform). The limit of quantification (LOQ) (signal-to-noise ratio of 10) for AOH, AME, ALT was 5 ng/g and for TeA 25 ng/g of wheat/rape seeds, 15 ng/g and 75 ng/g of lentils and 1.5 ng/ml and 7.5 ng/ml of grape fresh juice, must and wine respectively.

No immunochemical methods, e.g. enzyme-linked immunosorbent assay (ELISA), have yet been developed for AOH, AME, ALT, ATX-I and TeA in foodstuffs (V. Ostry, personal communications).

6. Occurrence of *Alternaria* mycotoxins in foodstuffs

Occurrence of *Alternaria* mycotoxins in foods and foodstuffs was last reviewed by Scott (2001). AOH, AME and TeA were frequently detected in apples, apple products, apple juice concentrates, mandarins, olives, pepper, red pepper, tomatoes, tomato products, oilseed rape meal, sunflower seeds, sorghum, wheat (as cited by Scott, 2001) and edible oils (olive oil, rapeseed oil, sesame oil, sunflower oil) (Kocher 2007). AOH and AME were detected in prune nectar, raspberries, red currant, barley, oats (as cited by Scott, 2001), Japanese pears (Tirokata *et al.*, 1969), citrus fruit (Magnani *et al.*, 2007) and carrots (Solfrizzo *et al.*, 2004, 2005). AME and TeA were detected in melon (as cited by Scott, 2001). AOH was found in blackberries, gooseberries and strawberries (as cited by Scott, 2001). Natural occurrence of AOH has been reported in apple juice, cranberry juice, grape juice, prune nectar, raspberry juice, red wine (as cited by Scott, 2001; Lau *et al.*, 2003; Scott *et al.*, 2006) and lentils (Ostry *et al.*, 2004). ATX-I and ATX-II were detected in *Alternaria*-infected apples, sorghum and wheat (as cited by Scott, 2001). The maximum levels of *Alternaria* mycotoxins reported in marketed products were in the range $1\text{-}10^3$ µg/kg; higher levels were found in

Table 3. Overview of the natural occurrence of AOH, AME, ALT and TeA in foodstuffs (recent studies).

Country	Foods/ Foodstuffs	Year	Mycotoxin (µg/kg)	n[a]	n+%[b]	Mean (µg/kg)	Range (µg/kg)	LOD/LOQ (µg/kg)	References
Argentina	tomato puree	2006	AOH	80	6	141	187-8,756	5[c]	Terminiello *et al.*, 2006
			AME	80	26	157	84-1,734	2[c]	
			TeA	80	29	227	29-4,021	11[c]	
Czech Republic	tomato puree	2006	AOH	10	100	7.9	1.2-14.1	0.08	J. Hajslova (personal communications)
			AME	10	100	1.3	1.2-2.7	0.05	
			ALT	10	10	0.4	N[d]	0.27	
Czech Republic	peeled tomato	2006	AOH	5	100	1.2	0.5-1.9	0.08	
			AME	5	100	0.2	0.2-0.3	0.05	
			ALT	5	0	-	-	0.27	
Czech Republic	tomato juice	2006	AOH	2	100	0.4	0.1-0.7	0.08	
			AME	2	50	0.8	N[d]	0.05	
			ALT	2	0	-	-	0.27	
Czech Republic	tomato ketchup	2004	AOH	8	100	6.9	0.3-27.4	0.08	
			AME	8	100	1.6	0.2-5.8	0.05	
			ALT	8	12.5	1.2	N[d]	0.27	
Czech Republic	tomato ketchup	2006	AOH	21	81	1.0	0.1-3.7	0.08	
			AME	21	100	0.4	0.06-1.2	0.05	
			ALT	21	0	-	-	0.27	
Canada	red wine (home)	2006	AOH	17	76	0.98	0.03-5.02	0.01	Scott *et al*., 2006
			AME	17	76	0.09	0.01-0.23	0.01	
	red wine import	2006	AOH	7	100	4.7	0.27-19.4	0.01	
			AME	7	26	0.06	0.01-0.19	0.01	
Canada	ice-wine (home)	2007	TeA	26	0	-	-	70	Abramson *et al*., 2007
Czech Republic	wine (home)	2004	AOH	39	0	-	-	1.5[c]	Ostry *et al*., 2007
	grape juice, must		AME	39	0	-	-	1.5[c]	
			ALT	39	0	-	-	1.5[c]	
			TeA	39	0	-	-	7.5[c]	
Germany	edible oils	2007	AOH	38	N[d]	N[d]	max 15	0.07	Kocher, 2006, 2007
			AME	38	N[d]	N[d]	max. 85	0.05	
			ALT	38	0	-	-	0.25	
			ATX-I	38	0	-	-	0.05	
			TeA	38	24	N[d]	max. 390	0.86	
			Tentoxin	38	26	N[d]	max. 44	0.11	
			AAL-toxins (TA-1 and TA-2)	38	0	-	-	0.14	
Czech Republic	wheat	2003-2005	AOH	129	46.5	7.7	6.3-44.4	5[c]	Skarkova *et al*., 2005
			AME	129	0	-	-	5[c]	
			ALT	129	91.5	18.7	6.3-41	5[c]	
			TeA	129	0	-	-	25[c]	
Sweden	barley, wheat, oats	2006	AOH	18	89	N[d]	9-335	N[d]	Häggblom *et al*., 2007
			AME	18	39	N[d]	1-184	N[d]	
			TeA	18	100	N[d]	980-4,310	N[d]	
Czech Republic	peas	2002-2003	AOH	84	0	-	-	3	Kralova *et al*., 2006
			AME	84	0	-	-	2	
			ALT	84	0	-	-	1	

[a] n = total samples
[b] n+% = percent of positive samples
[c] LOQ
[d] N = information not available

samples visibly infected by *Alternaria* rot, i.e. in products obviously not suitable for consumption. An overview of the natural occurrence of AOH, AME, ALT and TeA in foodstuffs is given in Table 3. The interpretation of these results for a conventional dietary exposure assessment and a probabilistic dietary exposure assessment of *Alternaria* mycotoxins in food is very difficult.

7. Conclusion

Monitoring of foods (e.g. total diet study) using LC-MS/MS method is desired in order to provide information on dietary intake of these toxins. Monitoring of foods may give impetus to further toxicological studies (for hazard characterisation e.g. of benchmark doses) if the occurrence of *Alternaria* toxins in food becomes a concern. There are currently no statutory or guideline limits set for *Alternaria* mycotoxins by regulatory authorities. Current data on the natural occurrence of *Alternaria* mycotoxins point to low human dietary exposure. Further information about occurrence and hazard characterization might lead to consideration for the need for regulations, however.

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