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The emerging Alternaria mycotoxin altertoxin II demonstrated substantial genotoxicity in vitro. Ubiquitous Alternaria ssp. frequently infest various agricultural crops, leading to economic losses and also potential food safety issues caused by associated mycotoxin contaminations. Due to the lack of commercially available reference standards, data on the general chemical behavior, the occurrence and the biological/toxicological effects of altertoxin II are scarce. Since tomatoes are particularly prone to Alternaria infestations, we simulated the storage and food processing of intact tomatoes and purees after altertoxin II-addition. We observed significant decrease in altertoxin II concentrations during storage at room temperature and particularly under thermal stress, by employing a validated LC-MS/MS method. Moreover, the reduction to the compound’s epoxide group to the alcohol, i.e. the formation of altertoxin I, was determined at considerable ratios in intact tomato fruits suggesting effective enzymatic xenobiotic metabolism.
The fate of altertoxin II during tomato processing at laboratory scale

Hannes Puntscher¹, Doris Marko¹, Benedikt Warth¹

¹Department of Food Chemistry and Toxicology, Faculty of Chemistry, University of Vienna, Währingerstr. 38, 1090 Vienna, Austria

CORRESPONDING AUTHOR: Benedikt Warth, University of Vienna, Department of Food Chemistry and Toxicology, Währingerstr. 38, 1090 Vienna, Austria.
Phone: +43 1 4277 70806
E-mail: benedikt.warth@univie.ac.at
ORCID: https://orcid.org/0000-0002-6104-0706
Abstract

The emerging *Alternaria* mycotoxin altertoxin II demonstrated substantial genotoxicity *in vitro*. Ubiquitous *Alternaria ssp.* frequently infect various agricultural crops, leading to economic losses and also potential food safety issues caused by associated mycotoxin contaminations. Due to the lack of commercially available reference standards, data on the general chemical behavior, the occurrence and the biological/toxicological effects of altertoxin II are scarce. Since tomatoes are particularly prone to *Alternaria* infections, we simulated the storage and food processing of intact tomatoes and purees after altertoxin II-addition. We observed significant decrease in altertoxin II concentrations during storage at room temperature and particularly under thermal stress by employing a validated multi-toxin LC-MS/MS method. For both, fresh and preheated tomato puree, between 87-90 % of ATX-II was recovered in samples kept at room temperature for 1.5 h, while in intact tomato fruits it was only 23%. However, only 2-4 % were recovered if the puree samples were heated to 100 °C for 30 min. Moreover, the reduction to the compound’s epoxide group to the alcohol, i.e. the formation of altertoxin I, was determined in intact tomato fruits (7-12 %) suggesting effective enzymatic xenobiotic metabolism.

Keywords

*Alternaria alternata*, emerging contaminants, food safety, liquid chromatography, tandem mass spectrometry, food processing, thermal treatment
1. Introduction

Altertoxin II (ATX-II) is a toxic secondary metabolite produced by the fungal genus *Alternaria* (Fig. 1). Also known as “black molds”, *Alternaria* spp. are ubiquitously occurring saprophytes and plant pathogens, often responsible for considerable economic losses due to infections of a broad variety of agricultural crops like cereals, tomatoes, and oil seeds (EFSA, 2016; Escrivá et al., 2017; Fraeyman et al., 2017; Lee et al., 2015; Ostry, 2008). Due to the capability of *Alternaria* fungi to produce toxic secondary metabolites, infected food and feed may imply a health risk for humans and animals. Moreover, in contrast to other molds endemic to rather warm climates, this genus can proliferate even at lower temperatures, allowing for infections not only on the agricultural field, but also post-harvest during refrigerated storage and transport (Juan et al., 2016; Ostry, 2008). The scientific report by the European Food Safety Authority released in 2016 (EFSA, 2016) elaborated a detailed dietary exposure assessment including the four most studied *Alternaria* toxins alternariol (AOH), alternariol monomethyl ether (AME), tentoxin (TEN) and tenuazonic acid (TeA). However, due to the lack of comprehensive occurrence and toxicological data of other emerging *Alternaria* toxins, a reliable risk assessment could not be conducted. Defining a threshold of toxicological concern (TTC value) of 2.5 ng/kg body weight per day for the genotoxic compounds AOH and AME, investigations indicated a possible health concern considering their estimated exposure data (EFSA, 2011, 2016). AOH and AME are regularly found in food commodities intended for human consumption (Hickert et al., 2017; López et al., 2016; Ostry, 2008; Puntscher et al., 2018; Tölgyesi et al., 2015; Walravens et al., 2016; Zwickel et al., 2016) and proved to be quite stable even along the food processing chain of tomato products (Estiarte et al., 2018), but also in fruit juices and wine (Scott and Kanhere, 2001) and even during bread baking (Siegel et al., 2010).

Interestingly, AOH plays only a minor role with the respect to genotoxicity of *Alternaria* culture extracts, while particularly ATX-II was identified to show a substantial genotoxic potential (Fleck et al., 2012; Schuchardt et al., 2014; Schwarz et al., 2012a). However, ATX-II has not been reported in naturally contaminated food samples so far. This might be due to the lack of commercially available reference material for the determination of ATX-II and the consequence that it is not screened for in standard assays. Only a few LC-MS based methods can determine and accurately quantify this potent toxin (Liu and Rychlik, 2015; Puntscher et al., 2018; Zwickel et al., 2016). To obtain reference standards it has been isolated from fungal cultures in these studies.

The genotoxic and mutagenic effects of AOH described *in vitro* (Brugger et al., 2006) were linked to its activity as a topoisomerase I and II poison (Fehr et al., 2009). The mechanism of action related to the even more potent genotoxic ATX-II has not been elucidated so far. The rather reactive epoxide
functionality of ATX-II is likely to be involved in its toxicological effects. However, even altertoxin I (ATX-I, Fig. 1), structurally the same scaffold but lacking the epoxide group, was reported to be mutagenic to a certain extent in vitro (Schrader et al., 2006). While ATX-II did not show estrogenic effects in Ishikawa cells (Dellaflora et al., 2018), chemical degradation reactions of the compound were suggested in the presence of the anthocyanin delphinidin (Aichinger et al., 2018). Little is known about metabolic pathways of ATX-II. In several cell lines (Caco-2, HCT 116, HepG2 and V79), it has been reported that the epoxide group of ATX-II was reduced to an alcohol resulting in ATX-I (Fleck et al., 2014a; Fleck et al., 2014b). In contrast, ATX-I seemed not to be further metabolized in Caco-2 cells. Xenobiotic pathways also found for AOH and AME like hydroxylation (Burkhardt et al., 2011; Pfeiffer et al., 2008; Pfeiffer et al., 2007; Tiessen et al., 2017) or glucuronidation (Burkhardt et al., 2012; Burkhardt et al., 2009; Burkhardt et al., 2011; Pfeiffer et al., 2009), were not determined neither for ATX-I nor for ATX-II (Fleck et al., 2014b).

Please insert Fig. 1 here

In the study at hand, we aimed to gain insights into the fate of an artificial ATX-II contamination in intact tomato fruits and tomato purees during food processing at a laboratory scale. Given that tomatoes are frequently infected by the ubiquitously occurring plant pathogens Alternaria spp., the stability and persistence of this highly genotoxic compound are of general interest as ATX-II contamination might be a considerable, yet under-investigated health issue for consumers.

2. Material and Methods

2.1 Reagents, solvents and chemicals

ATX-II was isolated from Alternaria alternata cultures grown on rice by an optimized protocol based on Schwarz et al. (2012b) and confirmed by NMR. ATX-I was purchased from Romer Labs (Tulln, Austria). Methanol (MeOH), water and acetonitrile (all LC-MS grade) were purchased from Honeywell (Seelze, Germany) and the eluent additives ammonia solution (25 % in water, for LC-MS) and ammonium acetate (LC-MS grade) from Sigma Aldrich. For sample preparation Milli-Q water, MeOH (HPLC grade) and acetic acid (p.a.) from Sigma Aldrich (Steinheim, Germany) were used.

The stock solution of ATX-II (250 µg/mL in MeOH) was diluted for the preparation of the working solution (25 µg/mL MeOH) needed for the tomato puree experiments and the preparation of calibration solutions. A multi component calibration solution (also including alterperylenol (ALP) and stemphytoltoxin-III (STTX-III), both isolated from rice cultures) was used for external calibration of additional Alternaria toxins. All solutions were demonstrated to be stable during storage at -20°C and measurement at 10°C over up to 72 h.
2.2 Sample preparation

Cherry tomatoes were purchased from a retail market in Vienna, Austria, in May 2018. Twelve fruits, taken from the same truss, were checked for the absence of visible fungal infections to minimize the chance of natural contamination. The tomatoes were thoroughly rinsed with water and subsequently dried on paper towels. All experiments were performed in triplicate. The study design is presented in Fig. 2. Six randomly picked tomatoes ("Intact" tomato samples) were stored at room temperature until the start of the experiment. The remaining six tomatoes were cut into pieces using a scalpel on a petri dish and homogenized in 15 mL tubes at room temperature using a FastPrep-24 5G™ High Speed Homogenizer (MP Biomedicals Life Sciences Division Santa Ana, CA, United States). The resulting tomato purees were combined ("Puree" samples), before transferring 24 representative aliquots of 1 g to plastic tubes (15 mL, Sarstedt). Six of these tubes were heated up to 100 °C under constant magnetic stirring for 30 min ("Pre-heated puree" samples) using a water bath. As a solvent control, nine tubes were filled with 1 mL Milli-Q water ("Solvent" samples).

ATX-II was added to six intact tomato fruits ("Intact" tomato samples) by an injection of the stock solution using a pipette (35-45 µL per tomato fruit). For “puree”, "pre-heated puree" and "solvent" control samples, the diluted working solution (25 µg/mL) was used: twelve aliquots of "puree", all six "pre-heated puree" and nine "solvent" control samples were spiked with ATX-II. The added volume of ATX-II solutions was adjusted for both, tomato fruits and tomato puree samples, to reach a final concentration of 1 µg ATX-II per 1 g of sample. No ATX-II solution was added to the remaining six aliquots of "puree" samples at this point. Three of these were providing for blank matrix samples ("Blank" samples) to investigate potential natural contaminations. The other three blank matrix samples were spiked right before their extraction ("Spike" samples) to reach the same final concentration of ATX-II solution as the other samples. These samples were used to determine the concentration of ATX-II at the formal time point 0 h (considered as 100%). All samples (apart from the intact tomato fruits) were vortexed gently after the addition of ATX-II to allow for appropriate homogenization. Subsequently, six “puree” samples and three “solvent” control samples were heated up to 100 °C for 30 min after adding ATX-II. Mimicking the thermal processing step allowed for the investigation of thermal stability. The prepared samples were divided in two batches: batch 1, which was extracted 1.5 h after ATX-II addition, and batch 2, which was extracted after 24 h (see Fig. 2). Batch 1 included three “intact” tomato fruits, six “blank” samples including the three intended for spiking right before the extraction, three “puree” samples kept at room temperature, three “puree” samples heated after ATX-II addition, three “pre-heated puree” samples, as well as three “solvent”
control samples kept at room temperature and three heated after ATX-II addition. Batch 2 included the remaining samples.

2.3 Sample extraction

Tomato samples were extracted according to the sample preparation protocol described in Puntscher et al. (2018). Tomato puree and solvent control samples were directly extracted, while whole tomatoes fruits were chopped and homogenized before (same procedure as described for the preparation of the “puree” samples, see above). All homogenized samples (1.000 ± 0.005 g) were extracted by adding 5 mL extraction solvent (methanol/water/acidic acid, 79/20/1, v/v/v) and shaking for 60 min using an over-head shaker (Roto-Shake Genie, Scientific Industries, NY, USA). These extracts were subsequently diluted 1:1 with methanol/water (10/90, v/v), centrifuged at 20.000 rcf and 4 °C for 15 min and stored at -20 °C until LC-MS/MS measurement.

2.4 Mass spectrometric quantitation

LC-MS/MS analysis was performed on a high-performance liquid chromatography (HPLC) system (UltiMate3000, Thermo Scientific) coupled to a triple-quadrupole mass spectrometer (MS, TSQ Vantage, Thermo Scientific) applying a quantitation method validated for tomato matrix as described by Puntscher et al. (2018). Briefly, the mass spectrometric system was operated in multiple reaction monitoring (MRM) mode using negative electrospray ionization. The target analyte ATX-II was quantified by matrix-matched calibration. Therefore, blank matrix extracts were spiked with the ATX-II stock solution. The same solution was also used for the tomato samples in the experiment. Calibration solutions were prepared for final concentrations of 0.1, 0.3, 1, 3, 10, 30 and 100 ng/mL. For quality control, solvent-matched calibration solutions were also measured (diluted with 10% methanol in water). Furthermore, tomato matrix-matched multi-analyte solutions, including other perylene quinones (ATX-I, STTX-III and ALP) were injected at the beginning and the end of a measurement sequence. These served to quantify ATX-I in the unknown samples. Moreover, the general integrity of the instrument was confirmed by the frequent measurement of solvent blanks, and a reference standard mix of known small molecules before and after each sequence. Chromeleon™ Chromatography Data System Software (version 6.80 SR13 Build 3818), Xcalibur™ Software (version 3.0, Thermo Scientific), and TraceFinder™ (version 3.3) were used for instrument control, data acquisition and data evaluation, respectively.
Results and discussion

The detection and quantitation of ATX-II and ATX-I was conducted by LC-MS/MS analysis. The performed spiking experiment confirmed a satisfying extraction efficiency of 102-104 % for ATX-II in the tomato matrix. Natural contamination of Alternaria toxins has been excluded by the analysis of blank matrix extractions. The concentrations of ATX-II were decreasing in all prepared samples over time. Surprisingly, after 1.5 h at room temperature, the ATX-II levels were reduced very similarly to 87-90 % (Fig. 3) in both tomato puree types, e.g. the “puree” just homogenized at room temperature and the “pre-heated puree” (heated up to 100 °C for 30 min before adding ATX-II at room temperature), as well as in the “solvent” control samples. The comparable decrease in the solvent control samples suggests a generally limited chemical stability or reactivity of ATX-II at room temperature per se. This has also been reported by Zwickel et al. (2016). After 24 h at room temperature, the levels further declined to 47-49 % in the tomato puree samples (“puree”, “pre-heated puree”) and to 18 % in water (“solvent”). This might give an indication that interactions with the polar solvent water do not favor a stable condition of the compound. ATX-II seemed to degrade/react slower in tomato matrix, potentially related to stabilizing pH conditions or by matrix interactions.

Please insert Fig. 3 here

Generally, thermal stress reduces the enzymatic activities of living tissue, which includes the plant metabolism of xenobiotics. Nevertheless, the ATX-II concentrations for the puree kept at room temperature and the thermally processed “pre-heated puree” were almost identical after 1.5 and after 24 hours, respectively. This raises the question, whether the enzymatic activity in the puree homogenized at room temperature was also reduced significantly by the applied mechanical stress. To prevent or minimize thermal stress already during homogenization, a pause of three minutes was included between two short homogenization steps of 40 s. However, by disrupting the natural texture of the tomato tissue and even damaging cell structures, enzymes might be inactivated to some extent, too. Thermal processing of the tomato puree samples after ATX-II addition clearly led to the most efficient reduction of ATX-II (>95 %). These samples, heated up to 100 °C for 30 min right after adding ATX-II, contained just 4 % and 2.5 % of the added concentration after 1.5 h and 24 h, respectively. Surprisingly, this is not the case for ATX-II in the solvent control samples after 1.5 h, which were heated identically right after the ATX-II addition. These levels were much higher (31 %) and might indicate that interactions with the matrix can play a different role at higher temperatures by allowing for adsorption, absorption effects, as well as covalent binding. Finally, ATX-II levels in intact tomato fruits were reduced to 23 % after 1.5 h and therefore much more efficiently as in all other samples at room
temperature. After 24 h, less than 1 % of the added amount was recovered. This strongly indicates active plant metabolism as an effective tool to deal with the xenobiotic ATX-II. Interestingly, 7 % of the ATX-II amount added to the intact tomato fruit was recovered as ATX-I after 1.5 h and 12 % after 24 h (for these calculations the molar masses of the compounds were taken into account). This clearly suggests that the tomato tissue is capable of reducing the epoxide group of ATX-II to the corresponding hydroxyl-group of ATX-I. This metabolic pathway has already been reported in in vitro experiments in the human cell lines Caco-2, HCT 116, HepG2 and the Chinese hamster cell line V79 (Fleck et al., 2014a; Fleck et al., 2014b), but not in plant metabolism. However, de-epoxidation is also known as a metabolic detoxification pathway for other epoxide-holding mycotoxins, including the trichothecone deoxinivalenol (DON). De-epoxy DON (DOM-1) was identified as a product of intestinal or rumen microbe activity and detected in animal excreta (Pestka, 2010; Yoshizawa et al., 1986). In this case, the epoxide of DON is converted to a carbon-carbon double bond, instead of being reduced to the alcohol like in the case of ATX-II. While sulfation and glucuronidation of DON is known for the hydroxyl groups on positions C3 and C15 preserving the epoxide, glutathione conjugates were identified in naturally contaminated grain primarily linked via the epoxide group (Uhlig et al., 2016). Mammalian epoxide hydrolases were reported to play a major role in converting a large number of structurally different epoxides to the corresponding less reactive vicinal diols and are therefore considered as important detoxification enzymes (Decker et al., 2009). In an in vivo study, we conducted very recently, a complex Alternaria culture extract containing high concentrations of ATX-I and ATX-II (among other Alternaria toxins) was administered to rats via gavage. While ATX-I was recovered in both urine and fecal samples, ATX-II was not (Puntscher et al., 2019).

In the presented study, much smaller amounts (in the lower ng range) of ATX-I were also determined in other tomato matrix samples, but not in the solvent controls. As it was observed for ATX-II, ATX-I concentrations were nearly the same for the “puree” kept at room temperature and the “pre-heated puree” (0.7-0.9 ng/g sample after 1.5 h and 3.1-5.0 ng/g sample after 24 h). These amounts correspond to less than 0.1 % and 0.3 % of the added ATX-II. Heating the sample after ATX-II addition led to slightly higher ATX-I concentrations. They were similar for both time points (13.4-14.7 ng/g), corresponding to 1.3-1.4 % of the added ATX-II. These results suggest that the tomato matrix contains components catalyzing chemical reduction of the ATX-II epoxide. However, this conversion reaction is much slower compared to intact tomato tissue (by a factor of up to 100). Since not 100 % of the decreased ATX-II was converted to ATX-I, further decomposing products might be identified in the future, as neither alterperylenol, nor stemphytoltoxin-III was determined in any sample.
3. Conclusion and outlook

We demonstrated that concentrations of the highly genotoxic *Alternaria* toxin ATX-II added to tomato products are decreasing when mimicking food processing steps at a laboratory scale. Generally decreasing concentrations due to degradation/reaction over time was determined to be comparatively slow, but clearly a factor in both intact tomato fruits as well as processed tomato purees. Thereby, interactions with matrix components (chemical reactions, ad- or absorptions) may play a considerable role. Compared to the respective tomato samples, after 1.5 h at room temperature, lower concentrations of ATX-II were determined in the water control samples. However, when applying thermal stress, they were higher in the control samples, suggesting temperature-dependent matrix interactions between ATX-II and the tomato components. Interestingly, intact fruits showed a much higher efficiency in reducing ATX-II concentrations at room temperature (up to 100-times more efficient compared to homogenized or heated tomato products). This and the increasing ATX-I concentrations suggest effective enzymatic activities promoting the reduction of the epoxide group of ATX-II. The formation of ATX-I traces of up to 0.3 % was also determined for all other tomato based samples. However, intact tomato fruits clearly showed the most efficient conversion rates to up to 12 % after 24 h. In conclusion, our results indicate a limited persistence of free ATX-II in tomato based food commodities (particularly during heated food processing) and the presence of efficient enzymatic detoxification strategies in living tomato tissue. However, potential health concerns caused by degradation/reaction products cannot be excluded. Moreover, large-scale food surveys are required to investigate the occurrence of perylene quiones in food commodities.

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Conflict of interest

The authors declare no conflict of interest.

Abbreviations

ALP, alterperylenol; AME, alternariol monomethyl ether; AOH, alternariol; ATX-I, altertoxin I; ATX-II, altertoxin II; LC, liquid chromatography; MRM, multiple reaction monitoring; MS, mass spectrometry; STTX-III, stemphytotoxin III; TeA, tenuazonic acid; TEN, tentoxin;
Fig. 1 Chemical structures of the *Alternaria* toxins altertoxin-I and altertoxin-II

Fig. 2 Study design for investigating the fate of altertoxin II in tomato commodities, also considering mechanical and thermal processing
Fig. 3 Altertoxin I and II concentrations in the experimental tomato and solvent control samples.
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