

The fate of alvertoxin II during tomato processing steps at a laboratory scale

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27 **Abstract**

28 Among various agricultural crops, tomatoes are particularly prone to *Alternaria* infections, which are
29 frequently resulting in economic losses and mycotoxin contamination. To investigate potential health
30 concerns implied for consumers, we simulated the storage and food processing steps of intact and
31 blended tomatoes after addition of the highly genotoxic secondary metabolite alvertoxin II. We
32 observed a significant decrease in alvertoxin II concentrations in samples stored at room temperature
33 and particularly those undergoing thermal treatment by employing a validated LC-MS/MS method.
34 When kept at room temperature, 87-90 % of ATX-II was recovered after 1.5 h in raw tomato purees
35 and purees heated before ATX-II addition, and 47-49 % were recovered after 24 h. In intact tomato
36 fruits the recovery was 23 % after 1.5 h and <1 % after 24 h. In heated purees (100 °C for 30 min after
37 ATX-II addition), also only minor concentrations accounting for 2-4 % were determined. Moreover, the
38 reduction of the compound's epoxide group to the alcohol, i.e. the formation of alvertoxin I was
39 demonstrated in intact tomato fruits (7-12 %), suggesting enzymatic biotransformation of the
40 xenobiotic by the plant's metabolism.

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49 **Keywords**

50 *Alternaria*, emerging contaminants, food safety, liquid chromatography, tandem mass spectrometry,
51 food processing, thermal treatment

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53 **1. Introduction**

54 Altertoxin II (ATX-II) is a toxic secondary metabolite produced by the fungal genus *Alternaria* (Fig.1).
55 *Alternaria spp.* are ubiquitous saprophytes and plant pathogens, often responsible for considerable
56 economic losses due to infections of agricultural crops like cereals, tomatoes, and oil seeds (EFSA,
57 2016; Escrivá et al., 2017; Fraeyman et al., 2017; Lee et al., 2015; Ostry, 2008). Moreover, health risks
58 might be implied for consumers by related mycotoxin contamination. The ability of proliferation even
59 at lower temperatures allows for post-harvest infections during refrigerated storage and transport
60 (Juan et al., 2016; Ostry, 2008). However, temperature and humidity influence *Alternaria* growth and
61 toxin production (Patriarca et al., 2014). The European Food Safety Authority released a dietary
62 exposure assessment evaluating the four *Alternaria* toxins alternariol (AOH), alternariol monomethyl
63 ether (AME), tentoxin (TEN), tenuazonic acid (TeA). For the genotoxic AOH and AME, estimated
64 exposure levels indicated a possible health concern (EFSA, 2011, 2016). These toxins were also shown
65 to be chemically relatively stable during food processing (Estiarte et al., 2018; Siegel et al., 2010).

66 ATX-II is significantly more genotoxic than AOH (Fleck et al., 2012; Schuchardt et al., 2014; Schwarz et
67 al., 2012a), however, it has not been reported in naturally contaminated food so far. Due to the lack
68 of commercially available reference material, it is not included in standard assays. Only a few LC-MS
69 based methods allow to determine and accurately quantify this potent toxin, after having isolated the
70 compound from fungal cultures (Liu and Rychlik, 2015; Puntischer et al., 2019a; Puntischer et al., 2018;
71 Zwickel et al., 2016). While genotoxic and mutagenic effects of AOH described *in vitro* (Brugger et al.,
72 2006) were linked to topoisomerase I and II poisoning (Fehr et al., 2009), the mechanism of action
73 related to ATX-II has not been elucidated so far. The reactive epoxide of ATX-II is likely to be involved
74 in toxicological effects. However, even altertoxin I (ATX-I, Fig. 1), structurally the same scaffold but
75 lacking the epoxide, was reported to be mutagenic to a certain extent *in vitro* (Schrader et al., 2006).
76 While ATX-II did not show estrogenic effects in Ishikawa cells (Dellafiora et al., 2018), chemical
77 degradation of the compound was suggested in the presence of the anthocyanin delphinidin
78 (Aichinger et al., 2018). In several cell lines (Caco-2, HCT 116, HepG2 and V79), it has been reported
79 that the epoxide of ATX-II was reduced to an alcohol resulting in ATX-I, which seemed not to be further
80 metabolized in Caco-2 cells (Fleck et al., 2014a; Fleck et al., 2014b).

81 In this study, we investigated the fate of ATX-II in intact and homogenized tomato fruits during food
82 processing steps at a laboratory scale. Given that tomatoes are frequently infected by the ubiquitous
83 plant pathogens *Alternaria spp.*, the stability and persistence of this highly genotoxic compound are
84 of general interest. ATX-II contamination might be a relevant, yet under-investigated health issue for
85 consumers.

86 2. Material and Methods

87 2.1 Reagents, solvents and chemicals

88 ATX-II was isolated in-house from *Alternaria alternata* cultures grown on rice (Schwarz et al., 2012b),
89 and confirmed by NMR. ATX-I was purchased from Romer Labs (Tulln, Austria). Methanol (MeOH),
90 water and acetonitrile (ACN) were purchased from Honeywell (Seelze, Germany), and ammonia
91 solution (25 % in water), ammonium acetate (all for LC-MS), MeOH (HPLC grade) and acetic acid (p.a.)
92 from Sigma Aldrich (Steinheim, Germany).

93 ATX-II was dissolved in MeOH (250 µg/mL) and further diluted in the same solvent (25 µg/mL). A multi-
94 component calibration solution (including ATX-I, as well as alterperyleneol (ALP) and stemphytoxin-III
95 (STTX-III), both isolated from rice cultures) was used for external calibration of additional *Alternaria*
96 toxins. All solutions were demonstrated to be stable during storage at -20 °C and repeated
97 measurements at 10 °C over 72 h.

98 2.2 Sample preparation

99 Cherry tomatoes were purchased from a retail market in Vienna, Austria, in May 2018. The study
100 design is presented in Fig. 1. All experiments were performed in triplicates. Firstly, twelve fruits from
101 the same truss without visible fungal infections were rinsed with water. Six randomly picked tomatoes
102 (“intact tomato” samples) were stored at room temperature until the start of the experiment (i.e. the
103 addition of the toxin). The remaining six tomatoes were cut into pieces and homogenized using a
104 FastPrep-24 5G™ High Speed Homogenizer (MP Biomedicals Life Sciences, USA). Aliquots of the
105 resulting tomato puree (1 g each) were transferred to plastic tubes (15 mL, Sarstedt, samples A, B,
106 and, C). Six of these were heated at 100 °C for 30 min (“Pre-heated” puree samples, samples A) using
107 a water bath. Samples B and C were kept at room temperature in the meantime. As a solvent control,
108 nine tubes were filled with 1 mL Milli-Q water (“solvent control”, samples S).

109 ***Please insert Fig. 1 here***

110 ATX-II stock solution (250 µL/mL) was injected into six “intact tomato” fruits (35-45 µL per tomato
111 fruit). Therefore, a pipette tip was used to pierce the peel once and inject the solution into the fruit
112 (about 10-15 mm underneath the peel). For the samples A, B, and S, the ATX-II working solution
113 (25 µg/mL) was used. All ATX-II additions were adjusted to result in a final concentration of 1 µg ATX-
114 II per 1 g of each sample. No ATX-II solution was added to the samples C at this point. Three of these
115 were providing for “blank” matrix samples to investigate potential natural contamination. The other
116 three samples C were spiked after 1.5 h, right before extraction (“Spike” samples), providing
117 concentrations at the formal time point 0 h (considered as 100 %). All puree and solvent samples were

118 vortexed gently after the addition of ATX-II to allow for appropriate homogenization. Subsequently,
119 six samples B (B2 in Fig. 1) and three samples S (S2 in Fig. 1) were heated to 100 °C for 30 min,
120 mimicking a thermal processing step. Three “intact tomato” fruits, three samples A (“pre-heated”
121 puree), three samples B1 (“non-heated” puree), three samples B2 (“heated” after ATX-II addition), six
122 samples C (for blank and spiking), and six samples S (“non-heated” S1, “heated” S2) were extracted
123 after 1.5 h. The remaining samples were extracted after 24 h. The first time point was chosen to allow
124 comparison with data in literature (Aichinger et al., 2018; Fleck et al., 2014b), while the second was to
125 investigate subsequent progress and trends.

126 **2.3 Sample extraction**

127 All samples were extracted as described in Puntscher et al. (2018). Intact tomato fruits were chopped
128 and homogenized before applying the same procedure as for the preparation of the puree samples
129 (see above). All homogenized samples (1.000 ± 0.005 g) were extracted by adding solvent (5 mL,
130 methanol/water/acetic acid, 79/20/1, v/v/v) and shaking for 60 min using an over-head shaker (Roto-
131 Shake Genie, Scientific Industries, USA). These extracts were subsequently diluted 1:1 with
132 methanol/water (10/90, v/v), centrifuged (20.000 rcf, 4 °C, 15 min) and stored at -20 °C until
133 LC-MS/MS measurement.

134 **2.4 Mass spectrometric quantitation**

135 LC-MS/MS analysis was performed on a high-performance liquid chromatography (UHPLC) system
136 (UltiMate3000) coupled to a triple-quadrupole mass spectrometer (TSQ Vantage, Thermo Scientific)
137 applying a validated method (Puntscher et al., 2018) and Tracefinder™ (version 3.3) software for data
138 evaluation. Briefly, the mass spectrometric system was operated in multiple reaction monitoring
139 mode (MRM, quantifier/qualifier ions for ATX-II: m/z 349 -> 285/331, and for ATX-I m/z 351 ->
140 315/333) using negative electrospray ionization. Both albertoxins were quantified by matrix-matched
141 calibration (ATX-II: 0.1-100 ng/mL, ATX-I: 0.2-200 ng/mL). For quality control, standards in pure solvent
142 were included in the same sequence (10% methanol in water).

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144 **Results and discussion**

145 The detection and quantitation of ATX-II and ATX-I was conducted by LC-MS/MS analysis (Fig. 2 and
146 Table S1). The performed spiking experiment confirmed a satisfying extraction efficiency of 102-104 %
147 for ATX-II in the tomato matrix. Natural contamination of *Alternaria* toxins was excluded by the
148 analysis of blank matrix extractions. ATX-II concentrations were decreasing in all samples over time.
149 Surprisingly, after 1.5 h at room temperature, ATX-II levels were reduced very similarly to 87-90 % (Fig.
150 2) in both tomato puree types, e.g. the pre-heated samples A and the non-heated samples B1, as well
151 as in the "solvent control" samples S1. The comparable decrease in the solvent control samples
152 suggests a generally limited chemical stability or similar reactivity of ATX-II at room temperature *per*
153 *se*. This has also been reported by Zwickel et al. (2016). After 24 h at room temperature, the levels
154 further declined to 47-49 % in the tomato puree samples (A and B1) and to 18 % in S1. This indicates
155 that the polar solvent water is not favorable for stable conditions. ATX-II seemed to degrade/react
156 slower in tomato matrix, potentially related to stabilizing pH conditions or matrix-related interactions.

157 ***Please insert Fig. 2 here***

158 Despite the fact that thermal stress generally reduces enzymatic activity, ATX-II concentrations for the
159 thermally treated ("pre-heated") puree (A) and the non-heated puree (B1) were almost identical after
160 1.5 h and 24 h, respectively. This raises the question, whether xenobiotic metabolism was reduced
161 also by the applied mechanical stress during homogenization. Short blending steps of 40 s were
162 intended to minimize thermal stress applied to the matrix. However, the disruption of the tomato
163 tissue and cell structures might have inactivated enzymes to some extent. Thermal treatment of 30
164 min at 100 °C for the samples B2 (after ATX-II addition) clearly led to the most efficient reduction of
165 ATX-II (>95 %) indicating enhanced/accelerated reactivities. Only 4 % and 2.5 % of the added ATX-II
166 were recovered after 1.5 h and 24 h, respectively. Comparably higher ATX-II levels (31 %) were
167 determined in the heated solvent control samples (S2) after 1.5 h. Hence, ATX-II decrease may be
168 related to matrix interactions allowing for adsorption effects or covalent binding. Finally, ATX-II levels
169 in intact tomato fruits were reduced to 23 % after 1.5 h and therefore much more efficiently as in all
170 other samples at room temperature. After 24 h, less than 1 % of the added amount was recovered.
171 This strongly indicates active plant metabolism as an effective tool to deal with the xenobiotic ATX-II.
172 Interestingly, 7 % of ATX-II was recovered as ATX-I after 1.5 h and 12 % after 24 h (for these
173 calculations the molar masses of the compounds were taken into account). The tomato tissue seems
174 capable of reducing the epoxide group of ATX-II to the corresponding hydroxyl-group of ATX-I. This
175 metabolic pathway has already been reported in *in vitro* experiments in human and animal cell lines
176 (Fleck et al., 2014a; Fleck et al., 2014b), but not in plants. De-epoxidation is known as a detoxification

177 process for other epoxide-holding mycotoxins, including the trichothecene deoxynivalenol (DON)
178 (Pestka, 2010; Yoshizawa et al., 1986). Mammalian epoxide hydrolases were reported to play a major
179 role in converting a large number of structurally different epoxides to the corresponding less reactive
180 vicinal diols and are therefore considered as important detoxification enzymes (Decker et al., 2009).
181 In a recent *in vivo* study, a complex *Alternaria* culture extract containing high concentrations of ATX-I
182 and ATX-II (among other *Alternaria* toxins) was administered to rats *via* gavage. ATX-I, but not ATX-II,
183 was recovered in both urine and fecal samples (Puntscher et al., 2019b).

184 In the presented study, much smaller ATX-I amounts were also determined in other tomato samples,
185 but not in the solvent controls. As observed for ATX-II, ATX-I concentrations were nearly the same for
186 the puree samples A and B1 (0.7-0.9 ng/g after 1.5 h, 3.1-5.0 ng/g after 24 h, corresponding to less
187 than 0.1 % and 0.3 % of the added ATX-II). Heating of samples after ATX-II addition led to slightly higher
188 ATX-I concentrations (13.4-14.7 ng/g, corresponding to 1.3-1.4 % of the added ATX-II). Tomato matrix
189 components appear to catalyze chemical reduction of the ATX-II epoxide. However, the conversion
190 reaction in intact tomatoes is far more efficient (by a factor of up to 100). Since not 100 % of the
191 decreased ATX-II was converted to ATX-I, further decomposing products might be identified in the
192 future, as neither alterperyleneol, nor stemphylytoxin-III was determined in any sample.

193 **3. Conclusion and outlook**

194 We demonstrated that the concentrations of the highly genotoxic ATX-II added to tomato products
195 were decreased when mimicking food processing at a laboratory scale. Already at room temperature,
196 this *Alternaria* toxin was of limited stability in both, tomato puree and the solvent control water. No
197 notable difference was determined between tomato purees, which were heated before ATX-II
198 addition and those that were not. By thermal treatment of the contaminated puree, ATX-II levels were
199 significantly reduced, indicating increased matrix-related reactivity, possible adsorption, covalent
200 binding, chemical modification and/or degradation. Intriguingly, intact tomato fruits demonstrated a
201 more efficient reduction with <1 % ATX-II recovered after 24 h at room temperature. The conversion
202 of ATX-II to ATX-I by de-epoxidation (up to 12 % after 24 h) suggests effective plant detoxification.
203 Potential health concerns caused by ATX-II or its degradation/reaction products cannot be excluded.
204 Future studies should investigate the structure(s) of the latter as well as their toxicological potential.
205 Moreover, large-scale food surveys are required to investigate the occurrence of perylene quinones
206 in food commodities.

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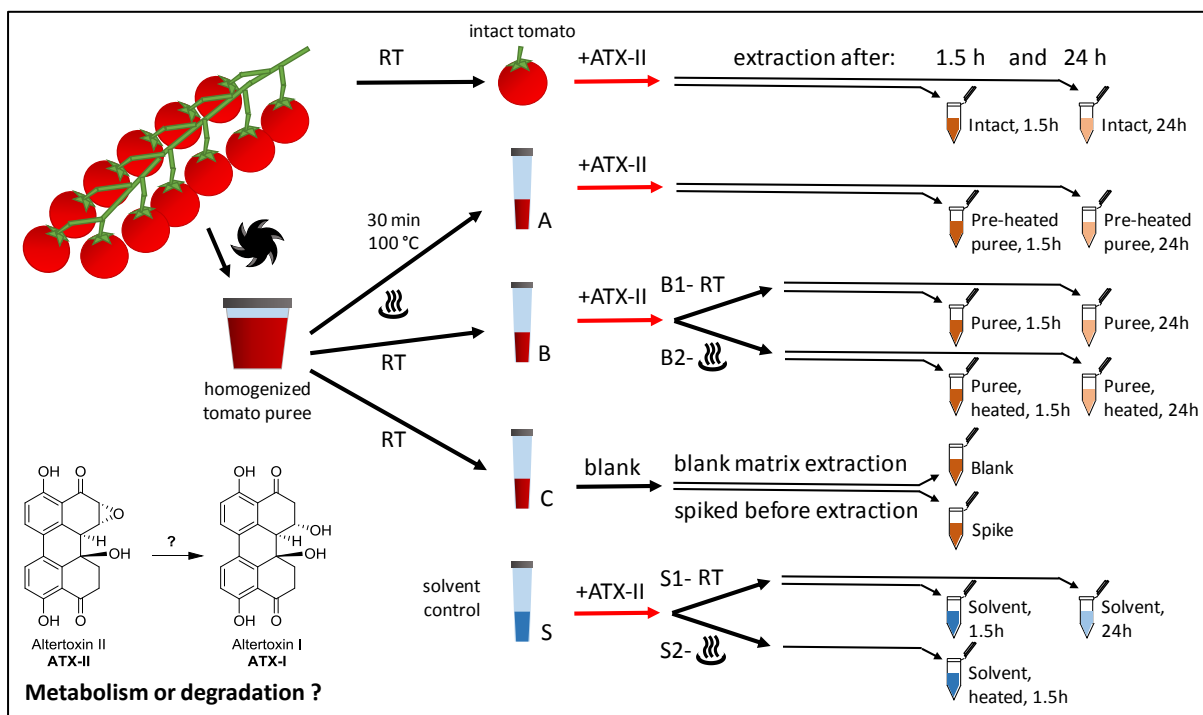
212 This manuscript has been released as a Pre-Print at ChemRxiv (Puntscher et al., 2019c).

213 **Conflict of interest**

214 The authors declare no conflict of interest.

215 **Abbreviations**

216 ALP, alterperyleneol; AME, alternariol monomethyl ether; AOH, alternariol; ATX-I, altertoxin I; ATX-II,
217 altertoxin II; LC, liquid chromatography; MRM, multiple reaction monitoring; MS, mass spectrometry;
218 STTX-III, stemphytoxin III; TeA, tenuazonic acid; TEN, tentoxin;

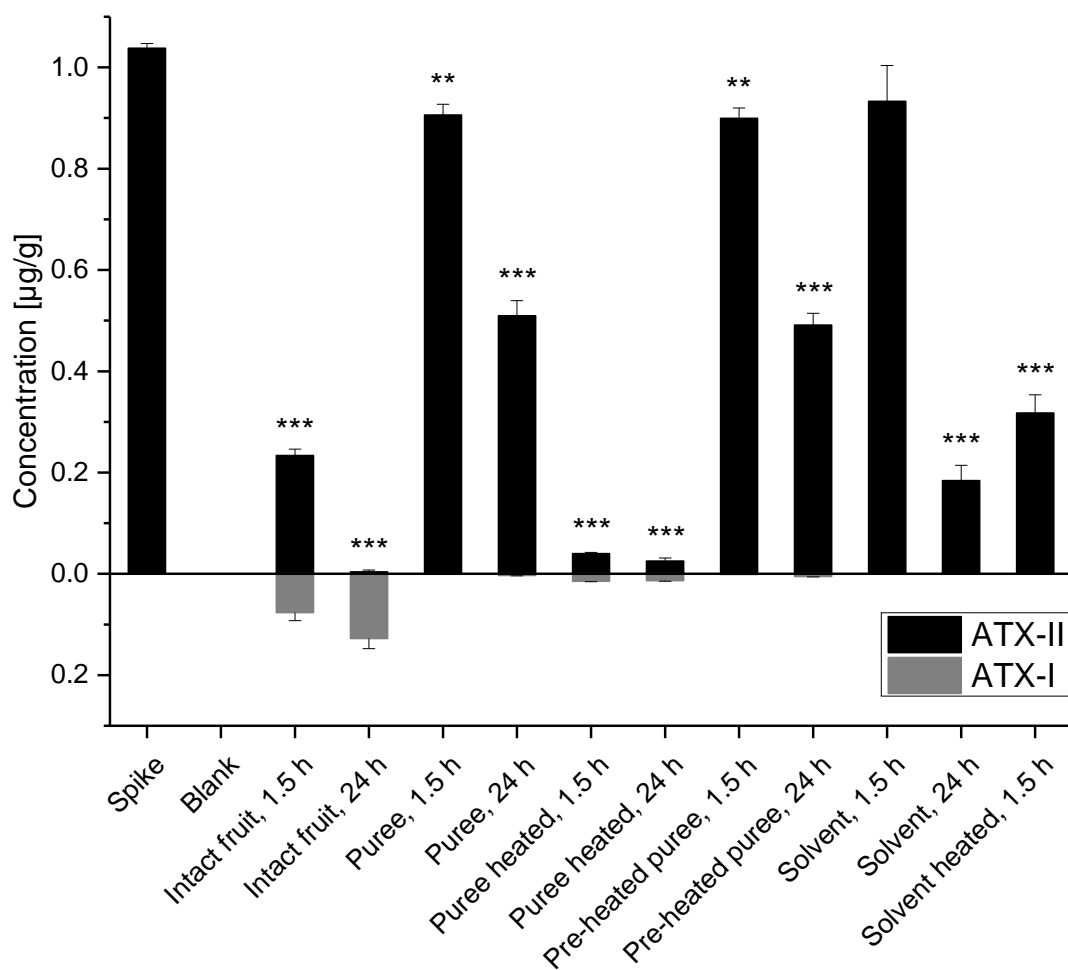


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222 **Fig. 1** Study design for investigating the fate of alvertoxin II in tomato commodities taking into account
 223 mechanical and thermal processing steps

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225
 226 **Fig. 2** Alternantoxin I and II concentrations in the experimental tomato and solvent control samples. Significant
 227 differences between the concentrations in the test samples and the “spike” samples were calculated applying
 228 an unpaired heteroscedastic Student's t-tests and are indicated by **($p < 0.01$) and ***($p < 0.001$)

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