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P11: DEVELOPMENT OF METABOLOMICS FOR *ALTERNARIA* TOXINS AS A MODEL FOR RISK ASSESSMENT OF MYCOTOXINS

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Abstract – A stable isotope dilution LC-MS/MS method was developed for the *Alternaria* toxins alternariol (AOH), alternariol monomethylether (AME) and tenuazonic acid (TeA) in herbs. Tentoxin (TEN) was quantified using matrix calibration. Solid phase extraction was applied for sample purification. Limits of detection (LOD) and quantification (LOQ), recoveries and intra- and interday precisions were determined for a comprehensive validation. The method was used to evaluate the mycotoxin contamination in various herbs and infusions.

Keywords: *Alternaria* toxins · SIVA · LC-MS/MS

1. INTRODUCTION

Alternaria toxins play an important role as "emerging" mycotoxins among food contaminants. Fungi of the genus *Alternaria* are ubiquitous plant pathogens, which produce several mycotoxins on fruits, vegetables and crops. As the mycotoxins AOH, AME, TeA and TEN are potentially harmful to humans the European Food Safety Authority (EFSA) estimated the relative risk due to dietary exposure of humans for these toxins by using the threshold of toxicological concern (TTC) approach. Toxicological data are missing to set a tolerable daily intake (TDI) for the *Alternaria* toxins [1]. For further risk evaluations, reliable and precise quantification methods for mycotoxins produced by *Alternaria* fungi in different food matrices are essential.

Siciliano et al. developed a LC-MS/MS method for the quantitation of AOH, AME, TeA, Altenuene (ALT) and TEN in rocket, cabbage and cauliflower. For quantification, matrix matched calibration was applied. LODs and LOQs ranged from 1.9 to 18.2 µg/kg as well as from 6.46 to 60.7 µg/kg. Recoveries were 51-102 %, respectively [2]. By means of using ^{13}C-labelled analogues as internal standards, Liu et al. developed a much more sensitive stable isotope dilution LC-MS/MS method for AOH, AME, alter-toxin I, altertoxin II and alterperylenol in grain, grain products and spices. Limits of detection ranged from 0.09 to 0.53 µg/kg. Recoveries were 96-109 %, respectively, and relative standard deviations of intra- and interday precisions were lower than 13 % [3].

The aim of this study was to develop and validate a sensitive LC-MS/MS method for the quantitation of *Alternaria* toxins in herbs. Alternariol, alternariol monomethylether and tenuazonic acid were quantified using stable isotope dilution assays with ^{13}C- and ^{2}H-labelled analogues as internal standards. Tentoxin was quantified using matrix-matched calibration. Limits of detection (LOD) and quantification (LOQ), recoveries and intra- and interday precisions were determined for a comprehensive validation. Finally, *Alternaria* toxin contamination in various herbs and infusions was determined.

2. EXPERIMENTAL

2.1 Chemicals and reagents

Reference materials of AOH, AME, TeA and TEN as well as formic acid (>95%) were obtained from Sigma-Aldrich (Steinheim, Germany). All other solvents and chemicals were purchased from VWR (Ismaning, Germany). The internal standards [$^{2}H_4$]-AOH and [$^{2}H_4$]-AME were synthesized as reported earlier [4], [5]. [$^{13}C_6$, ^{15}N]-TeA was prepared in our laboratory as published [6].

2.2 LC-MS/MS measurement

LC–MS/MS was performed on a Shimadzu Nexera X2 UPLC system (Shimadzu, Kyoto, Japan). LC-parameters for AOH, AME and TEN were adopted from Liu et al. [3]. For AOH, AME and TEN, a HyperClone BDS-C18 column (150 x 3.2 mm, 3 µm, 130 Å, Phenomenex, Aschaffenburg, Germany) with a C18-guard column (Phenomenex, Aschaffenburg, Germany) was used as stationary phase. Column oven temperature was kept at 30 °C. The binary gradient system consisted of (A) ACN/2-propanol/H_2O (17.5/17.5/65, v/v/v) and (B) methanol at a flow rate of 0.2 mL/min. The gradient started at 0 % B for the first 3 min, raised linearly from 0 % B to 100 % B during 19 min and maintained at 100 % B for 1 min. Thereafter, the mobile phase returned to 0 % B within 2 min and the system was equilibrated for 5 min before the next run. The injection volume was 10 µL. For TeA, a Gemini-NX C18 column (150 x 4.6 mm, 3 µm, Phenomenex, Aschaffenburg, Germany) with a C18-guard column (Phenomenex, Aschaffenburg, Germany) was used as stationary phase. Column oven temperature was kept at 40 °C. The binary gradient system consisted of (A) 5 mM ammonium formate (pH 9) and (B) methanol at a flow rate of 0.5 mL/min. The gradient started at 5 % B for 3 min, raised linearly from 5 % B to 100 % B during 5 min and maintained at 100 % B for 2 min. The mobile phase returned to 5 % B within 3 min and the system was equilibrated for 10 min before the next run. The injection volume was 10 µL.

The LC was interfaced with a triple quadrupole ion trap mass spectrometer (LCMS-8050, Shimadzu, Kyoto, Japan). It operated in the negative ESI mode for AOH, AME, TEN and TeA. Parameters for the interface were set as follows: nebulizing gas flow 3 mL/min, heating gas flow 10 L/min, drying gas flow 10 L/min, interface temperature 300°C, dilution line temperature 250°C, heat block temperature 400 °C, collision-induced dissociation gas 270 kPA and interface voltage 4 kV. Direct infusion of standard solutions (1 µg/mL) was used for optimizing parameters for each standard. The mass spectrometer operated in the scheduled multiple reaction monitoring (MRM) mode for MS/MS measurements. For quantification, the mass transitions m/z 257.30 (261.30) → 215.05 (219.05) for AOH ($[^2H_4]$-AOH) and m/z 271.30 (275.30) → 256.20 (260.20) for AME ($[^2H_4]$-AME) were applied. TEN and TeA ($[^{13}C_6,^{15}N]$-TeA) were quantified using mass transition m/z 413.30 → 141.05 and m/z 196.30 (203.25) → 139.00 (142.00). Lab Solutions software (Shimadzu, Kyoto, Japan) was applied for data acquisition.

2.3 Calibration and quantitation

For calibration functions, varying amounts of analyte (A) were mixed with constant amounts of internal standard (IS) in molar ratios between 0.05 and 10 (1:20, 1:10, 1:8, 1:6, 1:4, 1:2, 1:1, 2:1, 5:1, 10:1). For AOH and AME, the $[^2H_4]$-labeled isotopologues and for TeA, the $[^{13}C_6, ^{15}N]$-lableled isotopologue were used. After LC–MS/MS measurements, the calibration functions were calculated from molar ratios [n(A)/n(IS)] against peak area ratios [A(A)/A(IS)]. Calibration functions for AOH, AME and TeA were received using linear regression. Seven matrix calibration points were prepared for the matrix-matched calibration curve of TEN in a concentration range between 0.2 to 10 µg/kg. The matrix calibration curve was calculated from peak area [A(A)] against concentrations of the analyte [c(A)]. The contents of the analytes in herbs were either calculated by using the respective calibration function (AOH, AME, TeA) or for TEN by using the matrix calibration function.

2.4 Sample preparation

To 1 g herbs or tea, 0.5 g sodium chloride and the internal standards (30 µL of $[^2H_4]$-AOH (0.1 µg/mL), 100 µL of $[^2H_4]$-AME (0.01 µg/mL) and 90 µL of $[^{13}C_6, ^{15}N]$-TeA (0.1 µg/mL)) were added. After mixing with 10 mL of MeOH/ACN/H_2O (10/45/45, v/v/v, pH 3.5) the sample was sonicated for 30 min and centrifuged at 3000 rpm for 5 min. The supernatant was evaporated and 10 mL of H_2O (pH 5.5) were added. The suspension was vortexed (20 s) and purified with a C18-SPE cartridge. The cartridge was washed with 6 mL MeOH, conditioned with 6 mL H_2O (pH 5.5) and the sample was loaded onto the column. The sample was washed with 6 mL H_2O and the mycotoxins were eluted with 6 mL MeOH and 9 mL MeOH + 2% NH_4OH. The eluate was collected and evaporated to dryness. After reconstitution with 200 µL MeOH/H_2O (1/1, v/v),

the sample was membrane filtered (0.22 µm). Analysis was performed with LC-MS/MS.

2.5 Method Validation

Limits of detection (LODs) and quantitation (LOQs)

Herbs free of *Alternaria* toxins were chosen as a blank matrix. LODs and LOQs were determined as recommended by Hädrich and Vogelgesang [7]. The blank matrix was spiked with unlabeled mycotoxins at four different levels (0.1, 0.4, 0.7 and 1.0 µg/kg for AOH; 0.03, 0.12, 0.21 and 0.3 µg/kg for AME; 0.3, 1.2, 2.0 and 3.0 µg/kg for TeA). Each amount was spiked in triplicate. For TEN, quantified by matrix-matched calibration, the signal to noise ratio approach was applied for the determination of LOD and LOQ.

Precision: Blank matrix was spiked with varying amounts of mycotoxins to determine intra- (n=3) and interday (during n=3 weeks) precision of measurements.

Recovery: Different amounts of AOH, AME, TeA and TEN were added in triplicate to herbs free of mycotoxins (0.4, 0.7, 1.0 µg/kg for AOH; 0.12, 0.21, 0.3 µg/kg for AME, 3.5, 7.0, 10.0 µg/kg for TeA and 0.4, 0.7, 1.0 µg/kg for TEN) and analyzed as described previously.

3 RESULTS AND DISCUSSION

3.1 Sample purification

Sample purification, i.e. removing interfering matrix components, was essential before analyzing the sample by LC-MS/MS. Solid phase extraction was the method of choice to minimize contamination of the HPLC-column and to increase sensitivity of the LC-MS/MS instrument. Optimization of solid phase extraction for AOH, AME, TEN and TeA was challenging due to various polarity. To improve retention of TeA on C18, the pH was adjusted to 5.5. For quantitative elution of TeA from C18, we applied a second elution step with 9 mL methanol containing 2 % ammonia.

AOH, AME and TEN were baseline separated using a HyperClone BDS-C18 column. LC-MS/MS chromatograms of AOH, AME and TEN (see chromatogram A) as well as the appropriate stable isotopically labeled internal standards [$^2H_4$]-AOH und [$^2H_4$]-AME (see chromatogram B) are shown in Fig. 1.

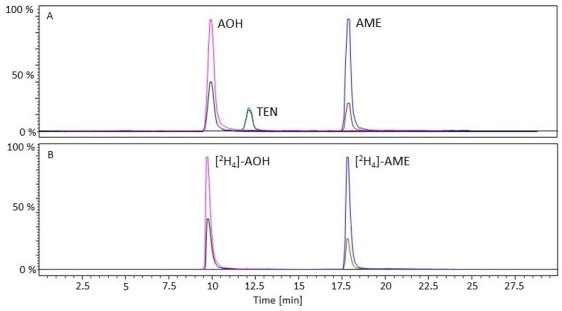

Figure 2: LC-MS/MS chromatograms of AOH, AME, TEN, [$^2H_4$]-AOH and [$^2H_4$]-AME

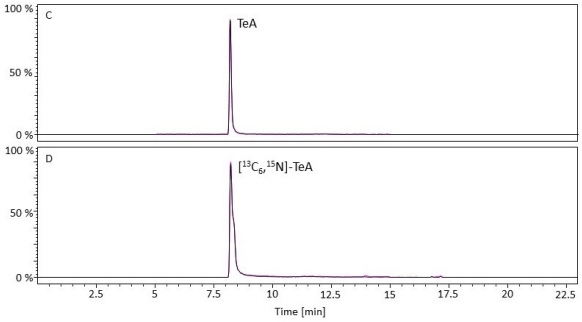

Figure 3: LC-MS/MS chromatograms of TeA and [$^{13}C_6$,^{15}N]-TeA

3.2 Method validation

Using herbs free of *Alternaria* toxins, LODs ranged from 0.02 to 0.45 µg/kg and LOQs from 0.06 to 1.4 µg/kg. Comparing our LODs and LOQs to the literature, we achieved very low limits of detection and therefore quantitation. Recoveries for AOH, AME, TeA and TEN at various concentrations were 97 to 114 %, respectively. Intra- and interday precisions of the analyzed mycotoxins varied from 4 to 5% as well as 6 to 10 %. Good recovery and low relative standard deviations of intra- and interday precisions confirmed robustness and precision of the developed method.

3.3 Analysis of mycotoxins in herbs and infusions

Different herbs and infusions were screened for *Alternaria* toxin contamination including parsley, dill, oregano, thyme, sage infusion, wild garlic, tarragon, melissa infusion, chive and bay leaves. We could not detect TeA in any of the analyzed samples, although, the LOD for TeA of the method is very low compared to the literature [2]. Four samples showed AME contamination, while AOH and TEN were quantified in just one sample. Sage infusion showed a contamination of 3.67 µg/kg AOH and 0.67 µg/kg AME. Melissa infusion contained 2.47 µg/kg AME and 1.63 µg/kg of TEN. Wild garlic and tarragon contained 0.15 µg/kg and 0.07 µg/kg AME. TeA was not detected in any sample. We could not detect any mycotoxin in parsley, dill, oregano, thyme, chive and bay leaves.

4 CONCLUSION

The developed stable isotope dilution LC-MS/MS method for *Alternaria* toxins enables sample purification of herbs and infusions by solid phase extraction without decreasing recovery of AOH, AME, TeA and TEN. Exact quantification of *Alternaria* toxins in herbs and other food matrices is essential for the risk assessment of the mentioned mycotoxins. To allow the EFSA to set up a TDI for *Alternaria* toxins, the developed mycotoxin method should be transferred and adjusted to other food matrices, especially to crops, vegetables, fruits and the appropriate processed products.

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