MULTI-MYCOTOXIN ANALYSIS OF *ALTERNARIA* MYCOTOXINS IN WHEET: CROSSTOX RECOVERY ASSESSMENT

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Abstract
A liquid chromatography-tandem mass spectrometry method for the determination of *Alternaria* toxins, including AME (Alternariol Monomethyl Ether), AOH (Alternariol) and TEN (Tentoxin) in wheat was validated using multi mycotoxin sample preparation. Nameli, the QuEChERS SPE method for mycotoxins – CrossTOX, which proved to give high recoveries for many mycotoxins. The recovery was assessed by analysis of spiked samples with a mixture of standard solutions of all three mycotoxins at two spiking levels (0.02 and 0.1 mg kg^{-1}) in six replicates. The obtained average recoveries and precisions (expressed as the RSDr, %) for Crosstox method were 102.1% (RSDr of 11.84%) for AME, 96.1% (RSDr of 16.10%) for AOH, and 95.9% (RSDr of 9.04%) for TEN. All the obtained recoveries were in accordance with the Commission Implementing Regulation (EU) 2023/2782.

Introduction
Alternaria species, ubiquitous fungal pathogens and saprophytes of the division Ascomycota, exhibit a remarkable ability to proliferate even under low-temperature conditions. These fungi have been isolated from diverse agricultural products, including fruits, vegetables, cereals and oilseeds. Of particular concern is *A. alternata*, a prolific producer of over seventy distinct secondary metabolites. The studies have elucidated the chemical structures of several of these metabolites, revealing their mycotoxic properties detrimental to both human and animal health [1]. *Alternaria* mycotoxins are classified into five structural groups: perylene quinones (altertoxins I-III [ATX I-III]), dibenzo-α-pyrones (alternariol monomethyl ether [AME], alternariol [AOH]), tetramic acid derivatives (tenuazonic acid [TeA]), *A. alternata* f. spp. *Lycopersici* toxins (AAL-toxins) and miscellaneous structures (tentoxin [TEN]). Notably, AME, AOH and TeA are the most significant contaminants, frequently found in the cereals and animal feed [2].
Mycotoxins are naturally toxic compounds with a low molecular weight and a high bioaccumulation ability and thermal stability which is very important for wheat and other cereals during the processing. According to literature, among more than 400 identified secondary compounds, deoxynivalenol, ochratoxin A, zearalenone and aflatoxins were renowned as the most studied mycotoxins and are considered a hazard to human or animal health [3]. *Alternaria* mycotoxins showed notably toxicity, such as mutagenicity, carcinogenicity, induction of DNA strand break, sphingolipid metabolism disruption, or inhibition of enzymes activity and photophosphorylation [4], which caused their massive research during past years.
Josef Hauptmann said: "Using the new CrossTOX® columns has made our mycotoxin multi-method much more effective, especially in terms of time and the use of consumables", indicate that this columns are good for the extraction of many mycotoxins including aflatoxins B1, B2,

G1, G2, sterigmatocystin, ochratoxin A, fumonisine B1 and B2, deoxynivalenol, nivalenol, 15-acetyl DON, 3-acetyl DON, DON-3-GLC, zearalenon, T-2 and HT-2, citrinin and diacetoxyscirpenol [5]. It was a reason to apply CrossTOX® in the analysis of Alternaria mycotoxins in wheat as part of the research.

Experimental

Reagents, solvents and equipment

The analytical standards of the AOH, TEN and AME were purchased from Romer Labs Biopure (Romer Labs Division Holding GmbH, Getzersdorf, Austria). The standards were reconstituted with 1 mL of the methanol to obtain 0.1 mg mL^{-1} stock solutions. All stock solutions were kept at 4 °C. The mixtures of all the toxins were prepared in acetonitrile (MeCN) in the final concentrations of 1 and 10 µg mL^{-1}. These solutions were used for spiking the blank samples for the recovery analyses.

The methanol and acetonitrile were LC-MS grade obtained from J.T.Baker. The ammonium formiate and acetic acid were analytical grade purchased from Merck (Darmstadt, Germany). The Zorbax Eclipse Plus C18 column Rapid Resolution HD (50x2.1mm, 1.8 µm particle size) and regenerated cellulose syringe filters (13 mm, 0.45 µm) were obtained from Agilent (Agilent Technologies, Inc, US). CrossTOX columns were obtained by LCTech (LCTech GmbH Obertaufkirchen, Germany).

The HPLC system was coupled to an Agilent 6475B LC/TQ triple quadrupole mass spectrometer with AJS ESI (Jet Stream Technology Ion Source). A Zorbax Eclipse Plus C18 column was used for the chromatographic separation. The column temperature was held at 35°C and the injection volume for the LC system was 2 µL. The chromatographic separation of the AOH, TEN and AME was carried out with mobile phase consisted of water (A) and acetonitrile (B), both containing 10 mM ammonium formiate, in a gradient mode and flow rate of 0,3 mL min^{-1}. A gradient elution started at 10% of B and composition was increased to 50% B at 3 min, 95% B at 6 min and held for 3 min. The composition of the mobile phase returned to the initial conditions in 1 min and the system was equilibrated during 3 min. The total running time was 10 min. The ESI source was used with the following settings: drying gas (nitrogen) temperature 220 °C, drying gas flow rate 10 L min^{-1}, nebulizer pressure 40 psi, sheath gas temperature of 250 °C, sheath gas flow 12 L min^{-1} and capillary voltage 3000 V. The detection was performed using the dynamic multiple reactions monitoring mode (dMRM). The Agilent MassHunter software (version 10.1 Agilent Technologies, 2006-2020) was used for the optimization and quantification.

The MRM mode was applied in the MS/MS detector and two ion transitions (quantifier and qualifier) were recorded for AOH, TEN and AME. The selected ion transitions with the optimized fragmentation (Frag) and collision energies (CE) are summarized in Table 1.

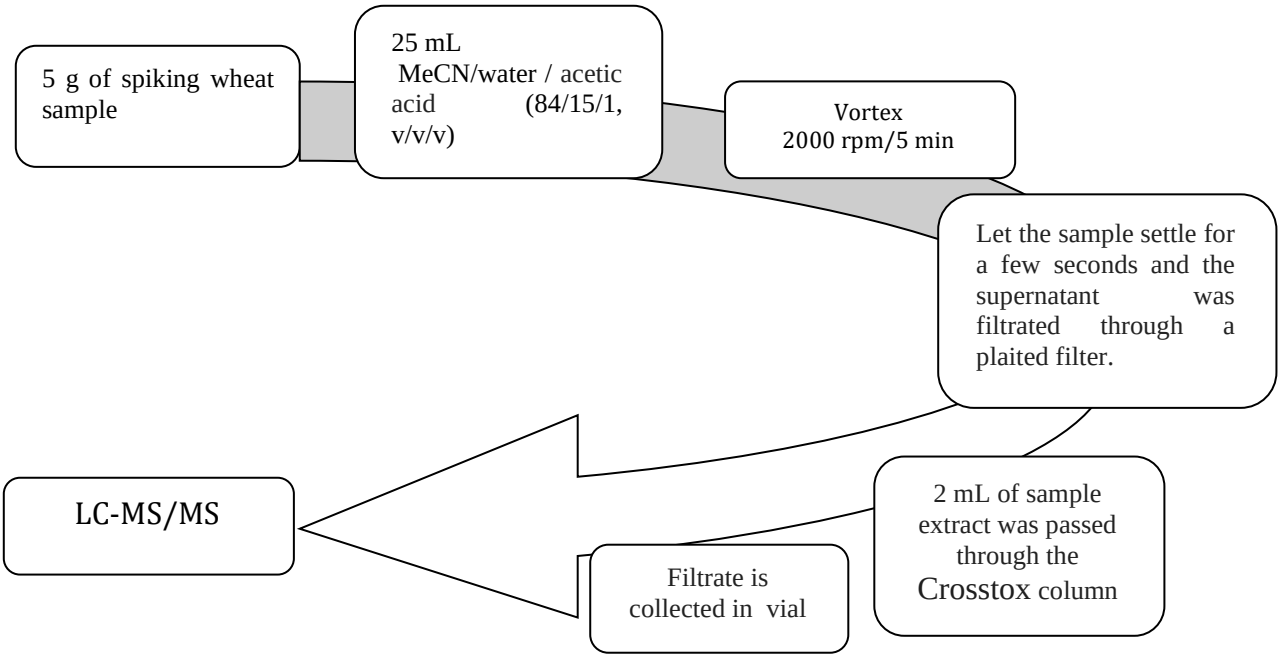
Table 1. Acquisition parameters for MS/MS determination

Compound	**Molecular mass**	**Precursor ion $[M-H]^-$ (*m/z*)**	**Product ions (*m/z*)**	**Frag (V)**	**CE (eV)**
AOH	256.0	257	215	120	23
			147		23
TEN	412.3	413	271	120	17
			215		23
AME	270.0	271	256	135	23
			228		30

Spiking samples and extraction

Homogenized blank wheat samples were spiked at two levels 0.02 and 0.1 mg kg^{-1} in six replicates. After spiking the blank samples, the extraction of the AOH, TEN and AME was performed (Figure 1).

Figure 1. Extraction of *Alternaria* mycotoxins

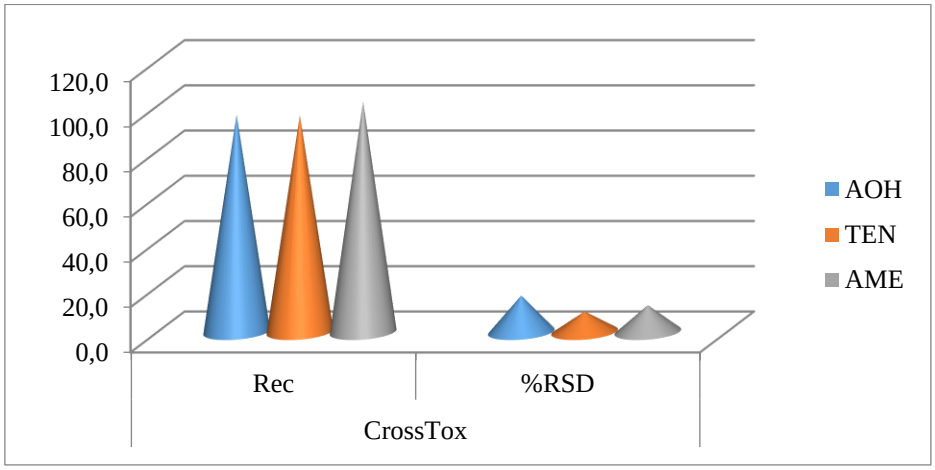


Results and discussion

The extraction of AOH, TEN and AME from wheat spiking samples using the CrossTox columns, proved to be a very simple, i.e. the described method for the extraction of the investigated mycotoxins uses a relatively small amount of organic solvents and thus protects the environment, while, on the other hand, it is not time consuming, which means that a large number of samples can be done in a short time.

The obtained average recoveries (Rec) and precision when the blank wheat samples were spiked at 0.02 and 0.1 mg kg^{-1} in six replicates are shown in Figure 2.

Figure 2. Rec (%) and RSD (%) of *Alternaria* mycotoxins



The obtained recovery values for all investigated *Alternaria* mycotoxins were in accordance with the Commission Implementing Regulation (EU) 2023/2782 (the average recovery should be between 70 and 120% and RSDr ≤ 20 %).

Conclusion

The wheat contributes 30% of the world's average crop consumption and is cultivated in most of the countries worldwide. According to the Republic of Serbia's statistical calendar, the estimated wheat production for this year is 2,901,000 tonnes, which is a 15.9% decrease compared to the last year's yield. Ensuring the wheat is free from contaminants, particularly mycotoxins, is crucial. The analysis of *Alternaria* mycotoxins using the CrossTOX method demonstrated the excellent recovery rates for AOH, TEN and AME.

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