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A "dilute-and-shoot" method for the *Alternaria* mycotoxins determination in wheat

Gorica Vuković[1], Vojislava Bursić[2*], Tijana Stojanović[2], Nikola Puvača[3], Dušan Marinković[2], Aleksandra Petrović[2], Bojan Konstantinović[2], Nataša Samardžić[2], Milena Popov[2]

[1]*University of Belgrade, Faculty of Agriculture, Nemanjina 6, 11080 Zemun, Serbia*
[2]*University of Novi Sad, Faculty of Agriculture, Trg Dositeja Obradovića 8, 21000 Novi Sad, Serbia*
[3]*University Business Academy in Novi Sad, Faculty of Economics and Engineering Management in Novi Sad, Department of Engineering Management in Biotechnology, Cvećarska 2, 21000 Novi Sad, Serbia*

**Corresponding author: bursicv@polj.uns.ac.rs*

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A B S T R A C T

Alternaria mycotoxins are the secondary metabolites for which the regulations, as well as the standardization, are yet going to be established by the European Commission and the European Committee for Standardization. This paper describes the possibility to apply a "dilute-and-shoot" method for the determination of tentoxin, alternariol and alternariol monomethyl ether in wheat. The used chromatographic method was liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). The strategy involves extraction the samples with the acetonitrile and water mixture (84/16, v/v), followed by the vortexing, centrifugation and filtration before the injection into the LC-MS/MS system. 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 "dilute-and-shoot" method were 76.3% (RSDr of 6.47%) for alternariol, 105.2% (RSDr of 2.16%) for tentoxin, and 86.0% (RSDr of 3.91%) for alternariol monomethyl ether. All the obtained validation data were in accordance with the Commission Decision 2002/657/EC and Commission Regulation (EC) No 401/2006. The main advantages of the present method are the simple and easy sample preparation, along with the high-sensitivity detection.

Keywords: *Alternaria* mycotoxins, dilute-and-shoot, LC-MS/MS, wheat.

И З В О Д

Alternaria микотоксини су секундарни метаболити плесни за које регулативе и стандардизација тек треба да буду установљени од стране Европске комисије и Европског комитета за стандардизацију. Ово истраживање представља могућност примене „разблажи-и-анализирај" методе за одређивање тентоксина, алтернатиола и алтернариол монометил етра у пшеници. Коришћена је хроматографска метода течне хроматографије са тандем масеном спектрометријом (LC-MS/MS). Процес подразумева екстракцију узорака мешавином ацетонитрила и воде (84/16, v/v), праћено вортексовањем, центрифугирањем и филтрацијом пре инјектовања у LC-MS/MS систем. Приликом одређивања приноса екстракције бланк узорци пшенице су обогаћени смешом стандардних раствора сва три микотоксина на два концентрациона нивоа (0,02 и 0,1 mg kg^{-1}) у шест понављања. Добијени приноси екстракције и прецизност (изражена као RSDr, %) за „разблажи-и-анализирај" методу су износили 76,3% (RSDr од 6,47%) за алтернариол, 105,2% (RSDr од 2,16%) за тентоксин и 86,0% (RSDr од 3,91%) за алтернариол монометил етар. Сви добијени резултати валидације су у складу са Одлуком Европске комисије 2002/657/ЕС и Уредбом Европске комисије (ЕС) 401/2006. Главне предности примењене методе су једноставна и лака припрема узорака, уз постизање високе осетљивости.

Кључне речи: *Alternaria* микотоксини, „разблажити и анализирај", LC-MS/MS, пшеница.

1. Introduction

During the 20th century the number of the introduced fungal infections in Africa and Europe was higher than the viruses and bacteria when combined (Puvača et al., 2020a). The mycotoxins represent the metabolites produced by the secondary metabolism of the fungi species. They are naturally produced in the food and feed products and may cause various toxic effects in humans and other vertebrates. The vertebrates usually get exposed to the mycotoxins by ingesting the contaminated food, predominantly grains and cereals (wheat, corn and peanuts) (Franco et al., 2018). These secondary metabolites may be produced under various climatic conditions and have been considered to be one of the most important public health hazards in the recent decades. Until now many of them have been determined in terms of chemical characterization and classification. For those mycotoxins which are considered to be the greatest

human and animal health hazards the maximum levels (ML) have been established, or the plans for the monitoring have been determined by the EU (Verstraete, 2019). Unfortunately, for some of them there is still not enough information considering their occurrence in order to calculate the exposure data, while for some of them the EFSA (European Food Safety Authority) accentuated the need for the action (Tolgyesi et al., 2015).

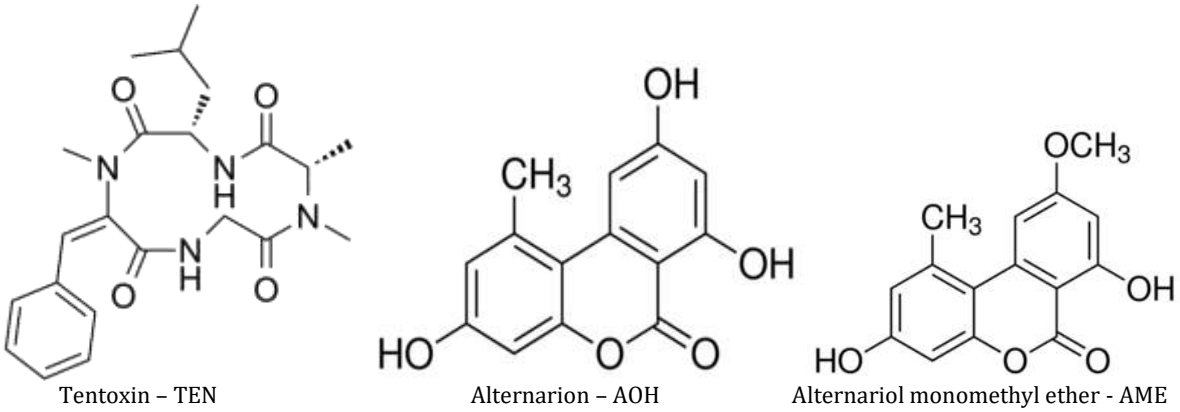
In case of the stored maize grains *Aspergillus, Fusarium* and *Penicillium* are the most common detected fungi genera (Krajaja et al., 2013). Some of the frequently detected fungi species in the food or feed in the Republic of Serbia are: *A. flavus*, *A. versicolor*, *F. oxysporum*, *F. solani*, *F. graminearum* and *P. aurantiogriseum* (=*P. verrucosum* var. *cyclopium*), with many of the species or strains still being undetermined (Udovički et al.,2018). Besides, the appearance of the mycotoxins of the genus *Alternaria* has been observed in Serbia, which is considered to be the consequence of the climate change, as well as the high adaptability and resistance of both saprobic and phytopathogenic *Alternaria* species (Đisalov, 2015). The *Alternaria* spp. significance is reflected not only in the deterioration of the wheat grain yield and quality, but also in the fact that many species of this genus produce toxic metabolites, which are harmful to human and animal health. A recent study by the EFSA dealing with the risks and the effects of the *Alternaria* mycotoxins on human and animal health speaks in favor of this scientific issue being topical (Đisalov, 2015). Mycotoxins produced by the *Alternaria* genus, such as alternariol (AOH), alternariol monomethyl ether (AME), altertoxin I (ATX-I) and II (ATX-II), altenuene (ALT), tenuazonic acid (TzA) and stemphyltoxin III (STTX-III), could be hazardous towards the animal health (Puvača et al., 2021).

Based on the available scientific data, which started to get published in recent years, the *Alternaria* mycotoxins have been determined in various matrices (Puvača et al., 2020). Scheibenzuber et al. (2021) dealt with the determination of thirteen *Alternaria* mycotoxins in beer. These secondary metabolites have also been investigated in tomato-based samples, in different flours, as well as the fruit and vegetable juices (Tolgyesi et al., 2021; Walravens et al., 2016). Gotthardt et al. (2019) investigated the presence of six *Alternaria* mycotoxins in infant food using stable isotope labeled standards.

Starting with the early discovery of the mycotoxins to the present day different analytical techniques have been used for their determination, with some of them being: thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) combined with different detectors (e.g., fluorescence, diode array, UV), liquid chromatography coupled with mass spectrometry (LC–MS), liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS), as well as the gas chromatography coupled with tandem mass spectrometry (GC–MS/MS), with the chromatographic techniques (specifically the LC-MS) being the most prevalent (Bursić et al., 2012; Agriopoulou et al., 2020). In order to determine multiple mycotoxins simultaneously the LC–MS/MS technique has been developed, and has drawn significant attention so far. However, when the rapid mycotoxins analysis is needed, the immunoassay-based methods take the lead, such as the biosensors, the lateral-flow devices (LFDs) and the enzyme-linked immunosorbent assay (ELISA) (Wang et al., 2022). The most recent techniques used for the mycotoxins determination in food are the molecular techniques, genomic and proteomic methods, as well as the electronic nose and hyperspectral imaging (HSI).

Today, some of the mycotoxins extraction techniques used for various matrices are: a liquid-liquid extraction (LLE), pressure liquid extraction (PLE), supercritical fluid extraction (SFE), solid phase extraction (SPE), matrix solid-phase dispersion (MSPD), ultrasound and homogenizing extraction with various organic solvents mixtures (Bursić et al. 2013, Vuković et al., 2017). The concept of the "Green Analytical Chemistry" evolved from the growing interest in developing more environmentally friendly chemical analyses (Breidbach, 2017). As the result of the mentioned concept, the QuEChERS method, which stands for "Quick, Easy, Cheap, Effective, Rugged, Safe", is regarded as the most current procedure for the extract purification, as well as the extraction (Capriotti et al. 2012). In the field of the mycotoxins research this method has drawn enormous attention because of how simple and effective it is for the mycotoxins isolation from the complex matrices, with also being environmentally friendly (Vuković et al., 2019).

Also, in order to perform the simultaneous mycotoxins analysis in food, the liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) was developed using the approach “dilute-and-shoot” (Sulyok et al., 2007).



Tentoxin – TEN Alternarion – AOH Alternariol monomethyl ether - AME

Figure 1. Chemical structures of *Alternaria* mycotoxins (Man et al., 2017; Scheibezuber et al., 2021)

The "dilute-and-shoot" method was used in the extraction of ohratoxins, fumonizins, zearalenon and deoxynivalenol from the corn meal, corn flour, wheat flour, rice and bean by Franco et al. (2018). Tolgyesi et al. (2021) used this method for the *Alternaria* mycotoxins determination in the tomato-based samples (tomato puree, ketchup and tomato sauce) and different flours (wheat, rye and maize). Sulyok et al. (2007) used this kind of extraction for the quantification of 87 analytes (mycotoxins) in food samples. All the "dilute-and-shoot" extraction methods are shown in Figure 2.

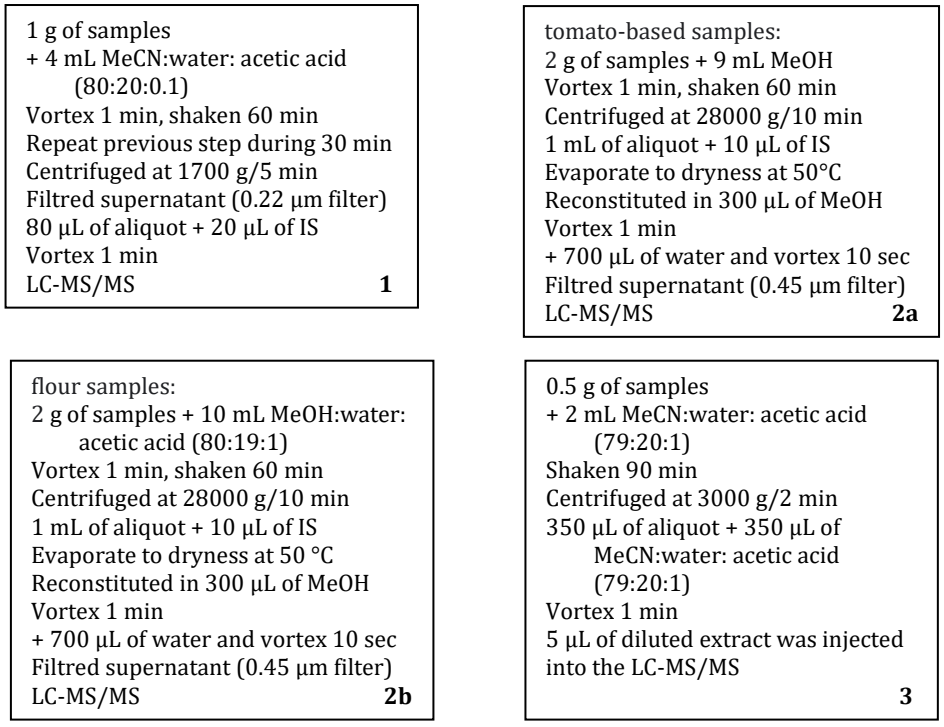


Figure 2. "Dilute-and-shoot" method (1-Franco et al., 2018; 2a, b- Tolgyesi et al., 2021; 3-Sulyok et al., 2007)

In the present study, the performance of a "dilute-and-shoot" LC-MS/MS analytical method was evaluated, aiming to the developed and validated method for the extraction and determination of the *Alternaria* mycotoxins (TEN, AOH and AME) in wheat. Wheat was chosen as a matrix because the grain production analyses emphasized that wheat and maize are the two most commonly grown crops in the Republic of Serbia (Grčak et al., 2020).

2. Materials and methods

2.1. Materials

2.1.1. Reagents, solvents and equipment

The analytical standards of the AOH, TEN and AME were purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands). The standards were reconstituted with 1.00 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 *Alternaria* toxins were prepared in acetonitrile (MeCN) in the final concentrations of 1 μg mL^{-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 Sigma-Aldrich. The ammonium formiate was 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 (15 mm, 0.45 μm) were obtained from Phenomenex (Utrecht, the Netherland).

The HPLC Agilent 1290 Infinity II chromatograph equipped with a quaternary pump, multisampler and column compartment thermostat was used for the *Alternaria* toxins detection. The HPLC system was coupled to an Agilent 6495 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 mobile phase consisted of water containing 0.1% ammonium formiate (A) and acetonitrile containing 0.1% ammonium formiate (B). The gradient elution conditions were as follows: 95% A (1 min), 60% A (1-7 min), 10% A (7-8 min) held for 2 min. Stop time was 1, with the equilibration of 2 min. The ESI sourse was used with the following settings: drying gas (nitrogen) temperature 200°C, drying gas flow rate 16 L min^{-1}, nebulizer pressure 30 psi, sheath gas temperature of 300°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 B.10.0 SR1 Agilent Tehnologies, 2006-2019) 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	257.0	215.2	120	25
			147.0		30
TEN	412.3	413.3	271.2	120	15
			215.1		20
AME	270.0	271.0	256.2	100	25
			228.2		30

2.1.2. 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 3).

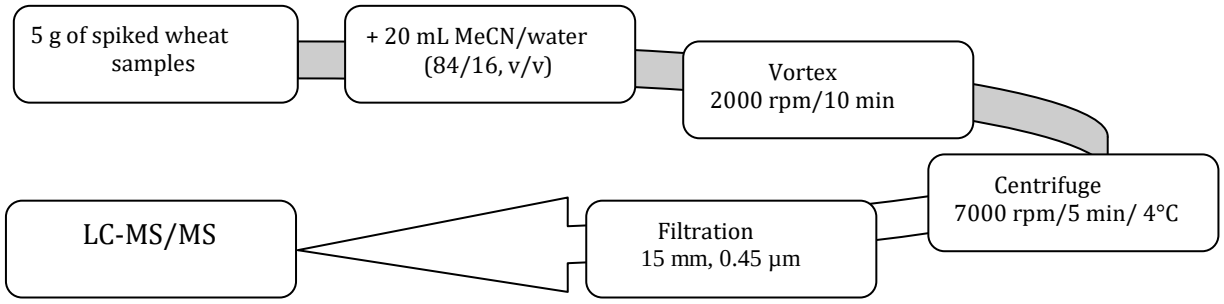


Figure 3. Extraction of *Alternaria* mycotoxins

3. Results and discussions

The multiple reaction monitoring mode (MRM) was used for the quantification and the confirmation of the *Alternaria* toxins (AOH, TEN and AME). All the obtained results for the recovery (Rec) and precision when the blank samples were spiked at two different concentration levels (0.02 and 0.1 mg kg^{-1}) in six replicates are shown in Table 2.

Table 2. Recovery data (%) and RSDr (%)

Mycotoxin	Concentration level (mg kg^{-1})	Replicates							
		1	2	3	4	5	6	Average	RSDr
AOH	0.02	81.3	74.5	75.4	71.2	60.1	70.6	72.2	6.43
	0.1	71.9	76.4	79.3	84.1	77.9	92.4	80.3	6.50
TEN	0.02	102.6	101.0	104.8	100.2	107.6	105.6	103.6	2.61
	0.1	107.1	103.8	109.2	106.3	108.1	105.8	106.7	1.72
AME	0.02	83.7	80.2	86.7	91.9	80.8	81.8	84.2	4.06
	0.1	87.5	83.8	84.2	88.4	95.2	88.1	87.9	3.75

The obtained average recoveries and precisions (express as RSDr, %) for "dilute-and-shoot" method were 76.3% (RSDr of 6.47%) for AOH, 105.2% (RSDr of 2.16%) for TEN and 86.0% (RSDr of 3.91%) for AME (Figure 4). All the obtained data were in accordance with Commission Decision 2002/657/EC and Commission Regulation (EC) No 401/2006.

There are so many different ways of mycotoxins extraction from different matrices which more or less successfully achieve satisfactory recoveries. Our research indicates a reliable and very simple way to extract three *Alternaria* mycotoxins (AOH, TEN and AME) from wheat.

The described "dilute-and-shoot" 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.

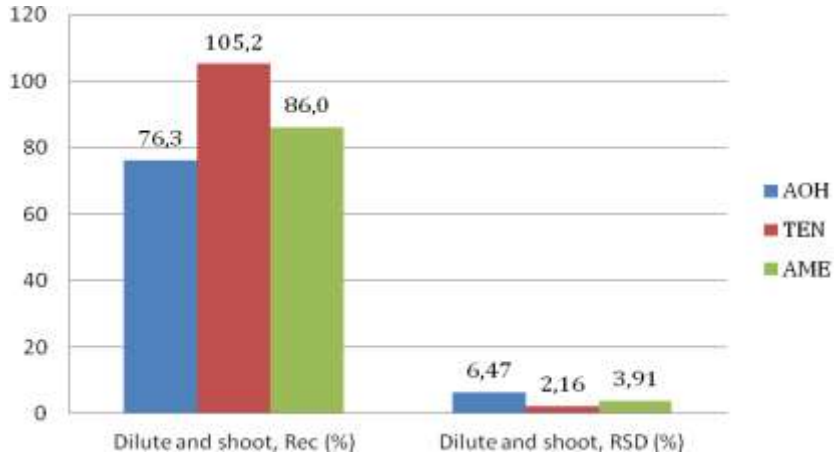


Figure 4. Rec (%) and RSD (%) graphical display

Comparing the obtained recoveries with the literature data values for all the *Alternaria* mycotoxins the conclusion is that they are in the range established by the Commission Regulation (EC) No 401/2006. Franco et al. (2018) for all the investigated mycotoxins and different matrices got the recoveries in the range from 89.0 to 108.0%, while the Sulyok et al. (2007) established the recoveries in the range from 70 to 110%. The validation results concerning the *Alternaria* mycotoxins recovery in the tomato-based products using the "dilute-and-shoot" method for AOH was in the range from 75.7 to 91.6% with the RSDr from 1.7 to 2.5%; for TEN recoveries was 87.3-88.7±2.6-5.5%, and for AME was in the range 67.6-74.6±1.0-3.5% (Tolgyesi et al., 2021).

4. Conclusions

A powerful instrument, such as the LC-MS/MS, can accept the "dilute-and-shoot" method with high sensitivity, thus avoiding cleaning and concentration steps. Considering the instrument which is used for the analysis, in comparison with the lowest targeter mass fraction, at least 10 times lower mass concentration of the analytes in the sample should be detected. The aim of this research was to exclude the SPE cleanup by using the dilute-and-shoot method for the analysis of the mycotoxins produced by the *Alternaria* genus (AOH, TEN and AME) in wheat as the matrix. Recently, we put the effort into developing "dilute-and-shoot" methods in order to analyze the *Alternaria* mycotoxins, which resulted in high recovery values for all the investigated toxins.

The "dilute-and-shoot" method was developed in order to analyze the *Alternaria* mycotoxins such as TEN – tentoxin; AOH – alternariol; AME – alternariol monomethyl ether in wheat. The method was validated only concerning one validation parameter – recovery, because this parameter best shows the efficiency of the sample preparation studied. Due to this approach, the sample preparation was fast, while the quantification was determined to be accurate. However, this strategy is limited by the necessity for the higher sensitivity of the LC-MS/MS instrument.

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