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Original research paper

THE CHOICE OF PREPARATION METHOD FOR THE DETERMINATION OF *Alternaria* TOXINS FROM WHEAT SAMPLES BY LC-MS/MS

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ABSTRACT: Cereals are the primary source of human diet, wheat being the third most produced grain worldwide, and in Serbia second most produced grain, just behind corn. As a result of climate change and global warming, frequent occurrences of mycobiota on steep grains can produce a negative impact on the safety of food products and their quality, which inevitably leads to large economic losses. Although *Fusarium* spp. remains a main source of mycotoxins contamination of wheat, in recent years, due to the evident climatic changes affecting agricultural production, other mycotoxigenic fungi have been pointed out as important wheat contaminants. Among them are the fungi of the genus *Alternaria,* especially *A. alternata*, which under favourable conditions, produces mycotoxins such as alternariol, alternariol monomethyl ether, tenuazonic acid, and other *Alternaria* toxins. Taking into account the toxicity of metabolites produced by certain species of fungi of the genus *Alternaria* in the system from farm to table, it is necessary to develop specific and sensitive analytical methods in order to implement systematic controls of occurrence of *Alternaria* toxins. Liquid chromatography coupled to (tandem) mass spectrometry (LC-MS/MS) has become the technique of choice for the detection and quantification of *Alternaria* toxins in food and feed. There are several limiting factors such as the efficiency of sample cleanup and the lack of reference materials for food and feed. The aim of this study was to to choose the most suitable preparation method for the determination of *Alternaria* toxins from wheat samples by LC-MS/MS technique based on published sample preparation methods, with possible modifications, which are used in analysis of mycotoxins. Modified method of wheat samples preparation by extraction with ethyl acetate was selected as acceptable based on extraction efficiency of analytes of interest.

Key words: *Alternaria toxins, preparation methods, wheat, LC-MS/MS*

INTRODUCTION

Alternaria toxins are mycotoxins produced by *Alternaria* species that cause plant diseases on many crops. They are widespread in both humid and semi-arid regions and can infect growing plants in the field. Toxin production is affected by interactions among *Alternaria* strain, the growing substrate and the environmental conditions (Barkai-Golan, 2008). *Alternaria* species are the principal contaminating fungi in wheat, sorghum and barley (Deshpande, 2002). Beside cereals, they have been reported to occur in oilseeds (sunflower and rapeseed), tomato, apples, citrus fruits, olives and several other fruits and vegetables, wine and beer (Scott and Kanhere, 2001; Scott, 2006; Ostry, 2008; Fernandez-Cruz et al., 2010; Scott et al.,

2012). *Alternaria* species produce more than 70 secondary metabolites, but a small proportion of these phytotoxins have been chemically characterised and reported to act as mycotoxins in humans and animals (Bottailico and Logrieco, 1992; Barkai-Golan, 2008). *Alternaria* toxins are divided into 5 different classes based on their chemical structures: (1) dibenzo-α-pyrones which include alternariol (AOH), alternariol monomethyl ether (AME), altenuene (ALT); (2) tenuazonic acid (TeA) and iso-tenuazonic acid (iso-TeA); (3), perylene quinones which include altertoxins I, II and III (ATX-I, ATX-II and ATX-III), and stemphyltoxin III; (4) AAL-toxins (AAL-TA and AAL-TB), abbreviation for *A. alternata* f. sp. *lycopersici* toxins; (5) a cyclic tetrapeptide such as tentoxin (TEN) (EFSA, 2011). From a toxicological point of view, there is strong evidence that AOH and AME could be mutagenic (An et al., 1989; Brugger et al., 2006). Although the acute toxicity of AOH and AME in mice is low (LD_{50}: 400 mg kg^{-1} bw), both compounds show remarkable cytotoxicity in cell cultures (Pero et al, 1973). Furthermore, it has been suggested that AOH and AME produced by *Alternaria alternata* on grain might be a factor responsible for the increased incidence of human oesophageal cancer in China (Liu et al., 1992; Pero et al., 1973). The methods of analysis of *Alternaria* toxins have been reviewed by Scott (2001) and more recently by Ostry (2008), Shephard et al. (2009, 2010, 2011, 2012, 2013), Köppen et al. (2010) and Berthiller et al. (2014). Several chromatography based techniques are suitable for *Alternaria* toxin quantification in foods and feeds, and liquid chromatography coupled to (tandem) mass spectrometry has become the method of choice for their determination. However, there are several limiting factors for the analysis of *Alternaria* toxins such as the efficiency of sample cleanup, the availability of (sufficient) amounts of standards and the lack of reference materials for food and feed. Most of the analytical methods are to a certain extent in-house validated but inter-laboratory validation studies, standardisation of the analytical methods or conduction of proficiency tests have not been reported (EFSA, 2011). Currently there are no regulations on *Alternaria* toxins in food and feed in Europe or in other regions of the world.

MATERIAL AND METHODS

Samples

Blank wheat sample free from the analytes of interest (TeA, AOH, AME) were collected from various growing regions in Vojvodina, Republic of Serbia.

Chemicals and reagents

Alternariol (AOH) (purity 99.0%), alternariol monomethyl ether (AME) (purity 99.5%), and tenuazonic acid (TeA) (purity 99.5%) were purchased from Sigma-Aldrich (Saint Louis, USA). Stock solutions of AOH, AME and TeA were prepared in methanol and stored at -20 °C. The following solvents were used: methanol (MeOH) (J.T.Baker, Deventer, The Netherlands) and ethyl acetate (EtOAc) (Sigma-Aldrich, Saint Louis, USA), all LC-MS grade, methanol (MeOH) and acetonitrile (ACN) (J.T.Baker, Deventer, The Netherlands), and dimethylformamide (DMF) (Sigma-Aldrich, Saint Louis, USA), all HPLC grade, formic acid (FA) (purity 99.9%,) (Carlo Erba, Italy), acetic acid (AA) (purity 99.8%), pa (lach - ner, Czech Repablic), fuming HCl (37%), pa, (Merck, Darmstadt, Germany), magnesium sulfate – anhydrous ($MgSO_4$) (purity ≥99.5%) (Sigma-Aldrich, Saint Louis, USA), sodium chloride (NaCl) (purity 99.9%) (Panreac, Barcelona, Spain). Deionized water was sourced from a Millipore Simplicity UV water purification system (Bedford, MA, USA).

Sample preparation

In order to overcome irregular mycotoxins distribution, sampling was performed according to EU requirements (2006/401/EC). Average blank wheat sample of approximately 10 kg was homogenized and quartered to obtain a 500 g of laboratory sample. Sample was ground to a 1 mm particle size using laboratory mill (Knifetec™ 1095 mill, Foss, Hoganas, Sweden).

The first method of sample preparation (Streit et al, 2013) was performed as follows: 5 g of homogenized wheat flour samples were extracted by shaking with

20 mL of acetonitrile/water/acetic acid mixture (79:20:1,v/v/v) for one hour, using an automatic shaker. Afterward, the suspensions were filtered and approx. 10 mL (exact volume known) of filtered crude extracts were transferred into glass cuvette, and evaporated under a stream of N_2 (Reacti-Therm I #18821, Thermo Scientific, USA). The dry residue was dissolved in 1 mL of DMF, and transferred to an HPLC vial through a 0.2 µm PTFE syringe filter (Econofilter, Agilent Technology, Germany).

The second method of sample preparation (Streit et al, 2013) was performed as follows: 5 g of homogenized wheat flour samples were extracted by shaking with 20 mL of acetonitrile/water/acetic acid mixture (79:20:1,v/v/v) for one hour, using an automatic shaker. Afterward, the suspensions were filtered and aliquots (1 mL) of filtered crude extracts were transferred into glass vials and diluted with 1 ml of mobile phase. Before injection into the liquid chromatography system, the extracts were passed through the 0.2 µm RC syringe filter.

The third method of sample preparation (Agilent application by authors: Reinhold and Bartels, 2010) was performed as follows: 20 g of the homogenized blank wheat flour samples were extracted by shaking with 60 mL of acetonitrile/methanol/ water mixture (pH 3; 45:10:45 v/v/v) for at least 2 min with an ultra-fast mixer. Then, they were centrifuged at 4000 rpm for 10 min and 6 mL of the supernatant was transferred to a centrifuge tube and was diluted with 15 mL of 0.05 M sodium dihydrogen phosphate solution (pH 3). The extract was cleaned up and concentrated by solid phase extraction using Bond Elut Plexa hydroxylated polymer cartridges (200 mg, 6 mL, Agilent Technology, US). After conditioning of cartridge with 5 mL methanol, followed by 5 mL of water, the diluted extract was passed through SPE tube. The centrifuge tube was rinsed with water and rinse loaded through the SPE tube. The SPE tube was then washed with 5 mL water, dried for 10 min under light vacuum and eluted with 5 mL methanol, followed by 5 mL of acetonitrile. Eluate was evaporated to dryness, reconstituted in 1 mL water/methanol (70:30, v/v), and passed through the 0.45 µm RC syringe filter.

The fourth method of sample preparation (Rasmussen et al, 2010) was performed as follows: A modification of the QuEChERS procedure (Annastassiades et al, 2003), used mainly for pesticide residue analysis, was employed for isolation of analytes. Briefly, 4 g of homogenous representative samples were weighted into the PTFE cuvette and 7.5 mL of 0.1% (v/v) formic acid and 10 mL of acetonitrile were added. The suspension was shaken vigorously for 3 min. After addition of 1 g NaCl and 4 g $MgSO_4$, the sample was shaken again, and it was centrifuged (5 min, 5000 rpm) in order to separate aqueous and organic phases. A 0.5 mL aliquot of upper organic phase was diluted with deionized water in 1:1 (v/v) ratio, and it was passed through the 0.2 µm nylon filter.

The fifth method of sample preparation (Siegel et al, 2010) was performed as follows: In each case approx. 1 g (exact weights known) of homogenized samples were mixed with 7 mL water. Subsequently, 2 mL of 2 M aq. HCl and 5 mL EtOAc were added. The resulting ternary phase systems were shaken for 45 min, ultrasonicated for 10 min (ATM40-3LCD, Madrid, Spain) and shaken again for 45 min. Then, the extracts were transferred into glass cuvettes and centrifuged at 5000 rpm for 15 min to achieve complete phase separation. Subsequently, 2 mL of the upper EtOAc layers were transferred into another glass cuvette, and evaporated under a stream of N_2 (Reacti-Therm I #18821, Thermo Scientific, USA). The dry residue was dissolved in 1 mL of LC/MC grade MeOH, and transferred to an HPLC vial through the Econofilter PTFE (13 mm, 0.2 µm) syringe filter (Agilent Technologies, China) and stored at -20 °C.

LC-MS/MS analysis

The Agilent 1200 series liquid chromatograph, consisting of vacuum degasser, binary pump, autosampler and thermostated column compartment was used for separation of analytes, whose detection was carried out by means of Agilent series 6410A triple-quad mass spectrometer with

electrospray ionization (ESI). MassHunter ver. B.03.01. software (Agilent Technologies) was used for instruments control and data analysis. The injection volume for all samples was 15 µL. The separation was achieved using a Zorbax Eclipce XDB-C18 column 50 mm×4.6 mm i.d., 1.8 µm (Agilent Technologies) with a column compartment temperature of 50 °C. The binary mobile phase consisted of 0.05% aqueous formic acid (A) and methanol (B) and was delivered at a flow rate of 1mL/min. Components were eluted in gradient mode, starting with 30% B, followed by a linear gradient reaching 70% B in 6 min, then by a linear gradient reaching 100% B in 9 min and holding for 3.0 min, with post-time of 3 min, and the entire effluent was transferred to mass spectrometer, without flow splitting. ESI parameters were as follows: drying gas (N_2) temperature 350 °C, flow 9 L/min, nebulizer gas pressure 45 psi, capillary voltage 4 kV. Compounds were quantified in negative ionization dynamic selected reactions monitoring mode. Each compound was monitored at determined retention time ± 1.5 min.

For the selection of preparation method for the determination of *Alternaria* toxins from wheat samples by LC-MS/MS in this study, recovery of the extraction step (R%), and possible matrix effects (ME) were determined for each sample preparation method. The selected parameters were evaluated by following the guidelines of Commission Decision 2002/657/EC. To evaluate the R% of three *Alternaria* toxins, blank samples were spiked with analyte standards at one concentration level, prior to extraction, and after extraction for the ME. The spiking level was 20 µg kg^{-1}, and samples were prepared in three replicates for each preparation method. The spiked samples were left at room temperature for half an hour in the dark. Thereafter, the samples were extracted, and stored at -20 °C until the analysis by LC-MS/MS. Five-point calibration curves for all mycotoxins were plotted at different concentrations. Linear regression was used to plot the peak area ratio (y) of each mycotoxin to its concentration. Each point was repeated in duplicate. The matrix effect (ME) and the recovery of the extraction step (R%) were calculated, using a protocol presented by Matuszewski, Constanez, and Chavez-Eng (2003) with the following formulas:

$$ME\ (\%) = B/A \times 100$$

$$R\ (\%) = C/B \times 100$$

where A is the average peak area in the standard solution, B is the average peak area in the spike after extraction, and C is the average peak area in the sample spiked before extraction.

RESULTS AND DISCUSSION

The aim of this study was to choose the most suitable preparation method for the determination of *Alternaria* toxins with acceptable level of recovery of the extraction step (R%), and minimal matrix effect (ME%) of target analytes from wheat samples. Also, existing methods were adapted to simultaneously analyse and quantify three *Alternaria* toxins (AOH, AME and TeA) in wheat samples, by using single clean-up procedure and analytical run.

Modification of the method for preparation of wheat samples by Streit et al. (2013) (first method) involved that the 10 mL (exact volume known) of filtered crude extracts were transferred into glass cuvette, and evaporated under a stream of N_2 in order to concentrate the target analytes and the dry residue was dissolved in 1 mL of DMF, and transferred to an HPLC vial through the 0.2 µm PTFE syringe filter, instead of diluting extract (ratio 1:1) and direct injection into the LC-MS/MS instrument. Mix of AOH, AME and TeA standards was also prepared in DMF in concentation range of 5 - 25 µg mL^{-1}. The second method of sample preparation was selected because this sample preparation method was used for the simultaneous detection and quantification of a broad spectrum of mycotoxins and other meta-bolites by a QTrap 5500 LC-MS/MS System (Streit et al., 2013). The third method of sample preparation was selected because, in this way prepared, tomatoes samples showed excellent recoveries for five *Alternaria* toxins (close to 100%) (Reinhold and Bartels, 2010). The modified QuEChERS procedure (fourth method) was selected since it is applied in the multi-mycotoxin (Rasmussen et al., 2010).

Table 1.
Molecular weight (MW), retention times (t_r), the m/z of precursor ion (Q1), m/z of monitored product ion (Q3), fragmentor voltage (FV), and collision energy (CE) of *Alternaria* toxins

Alternaria toxins	MW (g mol^{-1})	Dwell time (ms)	t_r (min)	Q1	Q3	FV (V)	CE (V)
AOH	258.2	0.1	4.92	257.2	215/213	180/180	25/20
AME	272.3	0.1	6.93	271.3	256/228	130/160	20/30
TeA	197.2	0.1	3.99	196.2	139/112	170/170	15/20

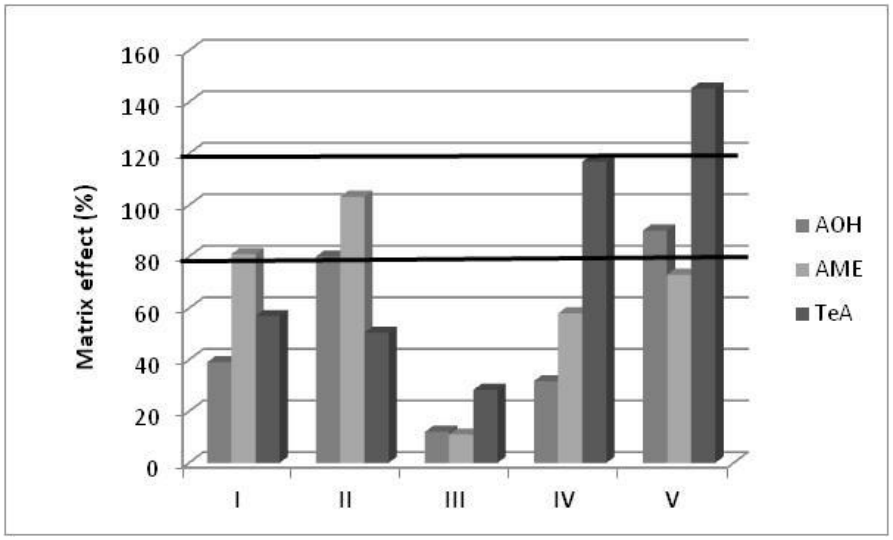

Figure 1. The influence of the sample preparation technique (I - by Streit et al. (2013); II - by Streit et al. (2013); III - by Reinhold and Bartels (2010); IV- by Rasmussen et al. (2010); V- by Siegel et al. (2010)) on the matrix effects of wheat sample on the response of each mycotoxin at concentration of 20 µg kg^{-1}. The tolerance level of matrix effect is shown between the two lines

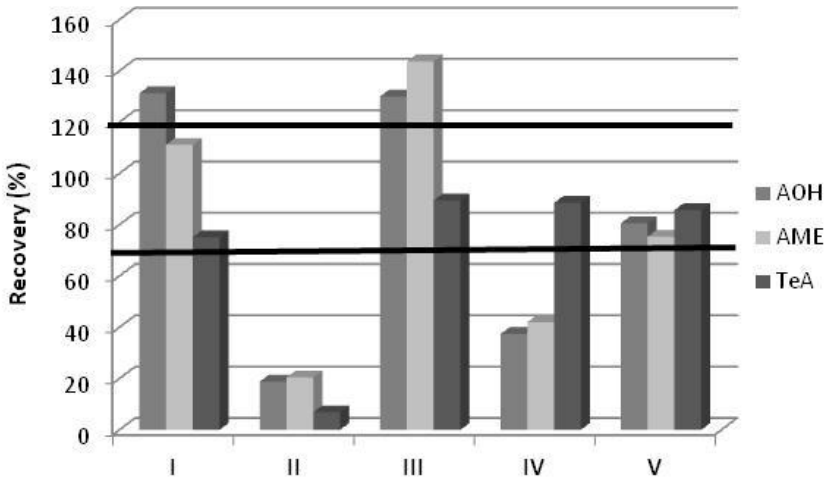

Figure 2. Recoveries of target analytes at 20 µg kg^{-1} level, using different methods of sample preparation (I - by Streit et al. (2013); II - by Streit et al. (2013); III - by Reinhold and Bartels (2010); IV- by Rasmussen et al. (2010); V- by Siegel et al. (2010)). The tolerance level of recovery is shown between the two lines

Modification of method for wheat sample preparation by Siegel et al., (2010) (fifth method) involved that the 2 mL of the upper EtOAc layer were transferred into another glass cuvette, and evaporated under a stream of N_2 in order to concentrate the target analytes and the dry residue was dissolved in 1 mL methanol, instead of direct injection into the LC-MS/MS instrument. Mix of AOH, AME and TeA standards was also prepared in methanol in concentation range of 5 - 25 µg mL^{-1}.

Ionisation efficiencies were evaluated on *Alternaria* toxins standard solution at 3000 µg kg^{-1} and the most intense characteristic MRM transitions were chosen for each analyte. Chromatographic and mass spectrometer data are summarized in Table 1.

Matrix effects are common problem that occurs when using LC–MS or MS/MS, and thus have an adverse effect on the analytical results. The response of the target compound can be enhanced or suppressed due to the interfering matrix components, which is commonly known as signal suppression/enhancement effect (SSE). A value close to 100% indicates that there is no significant matrix effect, while values >100% and <100% indicate signal enhancement and signal suppression, respectively. A range between (−20) and +20% ME or SSE in between 0.8 and 1.2 was considered as tolerable (Frenich et al., 2011; Yogendrarajah et al., 2013). Values outside this range indicate severe ME. The ME of different preparation methods on target analytes is shown in Figure 1.

It can be seen that the signal suppression effect was very prominent almost for all target analytes and almost for all applied methods of sample preparation. It is particularly strongly expressed in the third method of sample preparation (SPE). It is also remarkable that the fifth preparation method leads to a signal enhancement for TeA (ME>120%). High matrix effect was observed for target analytes in all tested preparation methods, suggesting that it is mandatory to perform matrix-mached calibrations.

Mean recoveries for all the tested preparation methods for the determination of three *Alternaria* toxins are shown in Figure 2.

It can be seen that the acceptable range of required performance criteria (EC 401/2006) of the mean recoveries for AOH, AME and TeA was observed using the fifth preparation method for determination of analytes of interest. Mean recoveries for AOH, AME and TeA were 81, 75 and 86%, respectively. On the other hand, the second sample preparation method does not provide a satisfactory recovery (extraction efficiency) for target analytes. In the case of the application of the first, third and fourth method of sample preparation, one or two analytes of interest does not meet the established criteria for recoveries (extraction efficiency).

CONCLUSIONS

Among all tested preparation methods for the determination of the content of three *Alternaria* toxins in wheat samples, the fifth method of preparation is shown to be satisfactory in terms of acceptable effectiveness of the extraction of the analytes of interest. Since this method shows significant matrix effects on the LC-MS/MS analysis, it is necessary to employ matrix-matched callibration to overcome these problems. Furthermore, complete validation of the method will be done for determination of the content of three *Alternaria* toxins in wheat samples by LC-MS/MS.

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ИЗБОР НАЧИНА ПРИПРЕМЕ УЗОРАКА ПШЕНИЦЕ ЗА ОДРЕЂИВАЊЕ *ALTERNARIA* ТОКСИНА ПРИМЕНОМ LC-MS/MS

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Сажетак: Жита представљају примарни извор људске исхране, при чему у светској производњи пшеница заузима треће место по произведеној количини, а у Србији заузима друго место, одмах иза кукуруза. Као последица климатских промена и глобалног загревања, интензивна појава микобиота на стрмим житима може произвести негативан утицај на безбедност прехрамбених производа, али и на њихов квалитет, што нужно доводи и до великих економских губитака. Иако *Fusarium* spp. и даље представља главни извор контаминације пшенице микотоксинима, у последљих неколико година због евидентних климатских промена које утичу на пољопривредну производњу дошло је и до појаве других токсигених гљива које су постале важни контаминенти пшенице. Међу њима истичу се гљиве из рода *Alternaria,* посебно *A. alternata* која под повољним условима продукује токсичне метаболите као што су алтернариол, алтернариол монометил етар, тенуазонична киселина и друге *Alternaria* токсине. Имајући у виду токсичност метаболита које продукују поједине врсте гљива рода *Alternaria* у систему од њиве до трпезе, за спровођење систематске контроле појаве *Alternaria* токсина неопходе су специфичне и осетљиве аналитичке методе. Течна хроматографија у спрези са масеним детектором (LC-MS/MS) постала је преовлађујућа техника за детекцију и квантификацију *Alternaria* токсина у храни и храни за животиње. Ограничавајући фактори су између осталог и понекад лоша ефикасност пречишћавања узорака и недостатак референтних материјала. Циљ овог рада био је да се изабере адекватан начин припреме узорака пшенице за одређивање садржаја *Alternaria* токсина применом LC-MS/MS технике на основу публикованих начина припреме узорака који се користе у аналитици микотоксина уз евентуалну модификацију истих. Од примењених начина припреме узорака пшенице модификована метода екстракције са етил ацетатом је одабрана као прихватљива у погледу ефикасности екстракције аналита од интереса.

Кључне речи: *Alternaria токсини, припрема узорака, пшеница, LC-MS/MS*

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