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Report on the development of a
method for the determination of
Alternaria toxins and citrinin in wheat,
tomato juice and sunflower seeds by
liquid chromatography – tandem mass
spectrometry

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by liquid chromatography – tandem mass spectrometry

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1. Executive summary

Alternaria toxins and citrinin are all mycotoxins produced by fungi growing on agriculture commodities. According to two recently published European Food Safety Authority (EFSA) reports these mycotoxins frequently appear in cereals, tomato, sunflower seeds and herbs; however, in the case of these mycotoxins no maximum levels (MLs) have been set yet. Legislative limits for *Alternaria* toxins and citrinin are currently under consideration by the European Commission.

In this study a new method has been developed for the determination of five *Alternaria* toxins and citrinin in wheat, tomato juice and sunflower seed samples based on a liquid chromatography – tandem mass spectrometric (LC-MS/MS) method. During the method optimization different high performance liquid chromatography (HPLC) columns packed with core-shell particles have been tested. The C-18 packing material has been finally used for the separation of mycotoxins. The sample preparation includes a solid-liquid extraction with pure methanol, followed by a derivatization step. Then, the samples are purified on polymeric-based solid-phase extraction cartridges.

The method performance characteristics have been evaluated by analysing spiked samples. The recoveries and repeatability vary from 63.3% to 113.1% and from 1.2% to 13.9%, respectively. The limits of quantification are between 1 and 10 µg/kg depending on the samples of interest.

Currently, the method is adapted to other LC-MS/MS instrument to demonstrate its transferability before in-house validation. Finally, the method is used for tomato juice proficiency test samples to evaluate its accuracy in naturally contaminated samples.

2. Introduction and aims

Mycotoxins are produced in a wide range of climatic conditions by fungi growing on agriculture commodities. Mycotoxins are the secondary metabolites of fungi. They have been considered as one of the biggest public health concerns worldwide for more than half a century [Zöllner & Mayer-Helm, 2006]. Most of them have already been chemically characterised and classified. Maximum levels (MLs) have been set for some mycotoxins in foods of plant and animal origin in the EU [Commission Regulation No 1881/2006, 2006]. However, there are still mycotoxins for which MLs are under consideration based on the present knowledge concerning their toxicity and occurrence. *Alternaria* species (e.g. *Alternaria alternata*) produce more than seventy secondary metabolites, of which only a few have been structurally identified and reported as hazardous to animals and humans [EFSA report, 2011]. Among the *Alternaria* toxins altenuene (ALT), alternariol (AOH), tentoxin (TEN), tenuazonic acid (TeA), and alternariol monomethyl ether (AME) are considered the most critical ones. *Alternaria* species can occur especially in cereals, oilseeds, tomatoes, apples, but also other fruits and vegetables. *Alternaria* mycotoxins are known to exhibit fetotoxic and teratogenic effects. Moreover, AOH and AME showed mutagenic and genotoxic properties [EFSA report, 2011; Ostry, 2008; Paterson & Lima, 2014; Van de Perre, 2014]. According to the European

Food Safety Authority (EFSA) agricultural commodities in Europe frequently contain ALT (73% of samples, maximum 41 µg/kg in wheat grains), AOH (31% of samples, maximum 1840 µg/kg in sunflower seeds), TeA (15% of samples, maximum 4310 µg/kg in oats), and AME (6% of samples, maximum 184 µg/kg in cereals) [EFSA report, 2011].

Besides *Alternaria* toxins, citrinin (CIT) is another important mycotoxin for which no ML has yet been set. The appearance of citrinin mainly in cereal-based products, beans, fruit & vegetable juices, and herbs is related to the agricultural commodities infected by *Aspergillus*, *Penicillium* and *Monascus* fungi. A recent EFSA report details the risks of CIT to human and animal health. The highest CIT concentrations detected in food (grain) and feed were 420 µg/kg and 998 µg/kg, respectively [EFSA report, 2012].

In this project a liquid chromatography – tandem mass spectrometric (LC-MS/MS) method for the simultaneous analysis of five *Alternaria* toxins (ALT AOH, TEN, TeA, and AME) and CIT has been developed. The method involves a solid-liquid extraction with methanol, and a subsequent derivatization for TeA. Then, samples are purified with solid-phase extraction on polymeric based cartridges, and finally, toxins are separated and quantified by LC-MS/MS. The objectives of the study were: (i) optimization of the LC-MS/MS parameters to achieve appropriate performance characteristics; (ii) development of a sample preparation that is suitable for a wide range of samples (e.g. wheat, tomato juice and sunflower seeds); (iii) evaluation of the method performance characteristics by analysing spiked samples.

Currently, the method is adapted to other LC-MS/MS instruments to demonstrate its transferability to a variety of instruments from different suppliers. This is however beyond the scope of the initial project.

3. Analytical method

3.1 Sample extraction

1.0 g sample is weight into 50 mL polypropylene (PP) centrifuge tubes, then 5.0 mL methanol is added and tubes are sealed. Samples are vortex-mixed for 5 s and horizontally shaken on a CAT S50 shaker at 600 min⁻¹ speed for 45 min at ambient temperature. Then, tubes are centrifuged at 2773 g for 10 min at 20 °C, and the upper layer is collected in a new 50 mL PP centrifuge tube. 100 µL derivatization reagent (0.596% dinitrophenylhydrazine in 2 mol/L HCl) is added to the sample and vortex-mixed for 5 s. The sample is let to be derivatized for 1 h at ambient temperature. Afterwards, 500 µL stop reagent (5% (v/v) undecanal in methanol) is added and vortex-mixed for 5 s. The sample is let to stand for 30 min and then diluted in the PP tube up to 35 mL with 50 mM ammonium formate buffer (pH 4, adjusted with formic acid). The sample is centrifuged at 693 x g (2000 rpm) for 10 min at 20 °C and subjected to solid-phase extraction (SPE) clean-up.

3.2 SPE clean-up

Strata-XL (200 mg, 6 mL, 100 μ m) cartridges must be conditioned with 6 mL methanol, followed by 6 mL water, and 6 mL 50 mM formate buffer. 75 mL reservoirs are connected onto the cartridges and samples are loaded into the reservoirs. Then, samples are passed through drop wise. Afterwards, SPE columns are washed with 6 mL methanol – water (15/85, v/v), and subsequently, with 6 mL n-hexane. Cartridges are vacuum dried for 5 min before eluting the samples into glass tubes with 5 mL methanol. Samples are evaporated to dryness at 45 °C under a gentle stream of nitrogen, and they are re-dissolved in 250 μ L methanol by vortex-mixing for 20 s. As a final step, samples are filtered through regenerated cellulose filters into HPLC vials.

3.3 LC-MS/MS analysis

Alternaria toxins and CIT are separated on an Ascentis Express C-18 (2.1 x 100 mm, 2.7 μ m) HPLC column equipped with a 2.1 mm C-18 precolumn using linear gradient elution. Two solvents (solvent A and B) are mixed by the binary pump. Solvent A contains 10 mM ammonium formate and 0.05% formic acid in water (pH 3), while solvent B was pure methanol. The flow rate is 0.3 mL/min. The methanol composition of the mobile phase is 20% at 0 min, 95% at 8 min, 95% at 12 min, 20% at 12.5 min. The total analysis time is 16 min. The column thermostat maintains at 30 °C and the injection volume is 5 μ L.

The HPLC system (Agilent 1100) is coupled to a MS/MS detector (Micromass Quattro Ultima PT) via an electrospray (ESI) interface that operates in negative mode. The optimized ESI settings are as follows: source temperature 125 °C, desolvation temperature 370 °C, drying gas flow 902 L/Hr, cone gas flow 76 L/Hr, capillary voltage -2.8 kV. Nitrogen is used as drying and collision gas (2.67×10^{-6} bar). Multiply reaction monitoring (MRM) mode is applied in the MS during the detection and two ion transitions are scanned for each target compound. The representative ion transitions with corresponding collision energies (CE) are as follows: CIT: 249.1 > 205.1 m/z (CE = 15 V) and 249.1 > 177.0 m/z (CE = 20 V); ALT: 291.2 > 202.9 m/z (CE = 30 V) and 291.2 > 248.1 m/z (CE = 20 V); AOH: 257.1 > 215.0 m/z (CE = 20 V) and 257.1 > 146.7 m/z (CE = 20 V); TEN: 413.5 > 271.2 m/z (CE = 15 V) and 413.5 > 214.8 m/z (CE = 15 V); TeA: 376.4 > 301.0 m/z (CE = 15 V) and 376.4 > 329.1 m/z (CE = 25 V); and AME: 271.1 > 256.2 m/z (CE = 20 V) and 271.1 > 228.2 m/z (CE = 20 V).

4. Method performance characteristics

Analytical method characteristics such as recovery, repeatability and limit of quantification were evaluated for wheat, tomato juice and sunflower seeds by analyzing spiked samples at 20 and 40 μ g/kg levels with four replicates each. For tenuazonic acid 200 and 400 μ g/kg fortification levels were selected because of the higher levels anticipated for future legislation based on the EFSA opinion. The LOQ was calculated from the signal-to-noise ratio (SNR) of the secondary ion transition

of a compound as ten times of SNR. Results are summarized in **Table 1**. In the case of wheat samples the recoveries were between 63.3% and 85.6%. The lowest recovery could be observed for CIT. The repeatability (RSD%) and LOQ was up to 11.3% and 5 – 10 µg/kg, respectively. For tomato juice samples the recoveries varied between 80.4% and 103.9%, and the RSD% was lower than 12.2%. The LOQ values ranged from 1 to 10 µg/kg. For sunflower seeds the recoveries and repeatability varied from 71.2% to 113.1% and from 2.8% to 17.9%, respectively. The LOQ was between 1 and 10 µg/kg.

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Table 1. Method performance characteristics. Recovery, repeatability (RSD%) and limit of quantification (LOQ).

Citrinin	Altenuene	Tentoxin	Tenuazonic acid	Alternariol	Alternariol monomethyl-ether
Wheat					
Recovery% (RSD%) at 20 – 40 ppb level	Recovery% (RSD%) at 20 – 40 ppb level	Recovery% (RSD%) at 200 – 400 ppb level	Recovery% (RSD%) at 200 – 400 ppb level	Recovery% (RSD%) at 20 – 40 ppb level	Recovery% (RSD%) at 20 – 40 ppb level
63.3% – 66.2% (8.2% – 8.9%)	73.1% – 76.0% (3.5 – 3.7%)	76.4% – 83.2% (1.2 – 6.5%)	73.1% – 76.5% (4.1 – 7.4%)	72.1% – 84.8% (2.3 – 11.3%)	79.2% – 85.6% (4.3 – 6.6%)
LOQ (µg/kg)	LOQ (µg/kg)	LOQ (µg/kg)	LOQ (µg/kg)	LOQ (µg/kg)	LOQ (µg/kg)
5	10	5	5	10	10
Tomato juice					
Recovery% (RSD%) at 20 – 40 ppb level	Recovery% (RSD%) at 20 – 40 ppb level	Recovery% (RSD%) at 200 – 400 ppb level	Recovery% (RSD%) at 200 – 400 ppb level	Recovery% (RSD%) at 20 – 40 ppb level	Recovery% (RSD%) at 20 – 40 ppb level
86.9% – 88.0% (7.5% – 12.2%)	82.5% – 86.0% (5.4 – 6.0%)	87.4% – 93.5% (6.3 – 6.8%)	97.7% – 103.9% (5.9 – 9.3%)	80.4% – 90.3% (7.1 – 8.5%)	89.9% – 93.9% (4.0 – 11.4%)
LOQ (µg/kg)	LOQ (µg/kg)	LOQ (µg/kg)	LOQ (µg/kg)	LOQ (µg/kg)	LOQ (µg/kg)
1	10	1	5	10	3
Sunflower seeds					
Recovery% (RSD%) at 20 – 40 ppb level	Recovery% (RSD%) at 20 – 40 ppb level	Recovery% (RSD%) at 200 – 400 ppb level	Recovery% (RSD%) at 200 – 400 ppb level	Recovery% (RSD%) at 20 – 40 ppb level	Recovery% (RSD%) at 20 – 40 ppb level
79.1% – 79.4% (10.0% – 10.5%)	82.1% – 85.8% (4.0 – 6.1%)	74.2% – 87.7% (2.8 – 17.9%)	107.2% – 113.1% (2.1 – 9.4%)	80.6% – 82.7% (3.1 – 13.9%)	71.2% – 71.6% (6.8 – 16.5%)
LOQ (µg/kg)	LOQ (µg/kg)	LOQ (µg/kg)	LOQ (µg/kg)	LOQ (µg/kg)	LOQ (µg/kg)
1	10	2	5	10	5

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Abstract

A new method was developed for the determination of five *Alternaria* toxins and citrinin in wheat, tomato juice and sunflower seed samples based on a liquid chromatography – tandem mass spectrometric determination. The optimized sample preparation involved a solid – liquid extraction with pure methanol, a derivatization step, and subsequent, solid-phase extraction purification on Strata-XL cartridges. The separation was carried out using HPLC column packed with core-shell C-18 particles. HPLC system was directly coupled to mass spectrometer via electrospray interface employed in negative mode. For the highest selectivity and sensitivity multiply reaction monitoring mode was used in the detector. The absolute recoveries (63.3% – 113.1%) and repeatability (1.2% – 13.9%) of the method, calculated from spiked samples, generated sufficient data.

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