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RESEARCH OPINIONS IN ANIMAL & VETERINARY SCIENCES

Liquid chromatography method validation for the simultaneous detection of deoxynivalenol, zearalenone, T-2 and HT-2 mycotoxins in vegetal products

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Abstract

Fusarium, Trichoderma, Stachybotrys and other fungal species are plant pathogens usually associated with cereals, which under certain environmental conditions can produce several secondary toxic metabolites. The major toxins found in cereals and cereal-based products such as deoxynivalenol, zearalenone, T-2 toxin and HT-2 toxin can be harmful to human and animal health. Liquid chromatography-tandem mass spectrometry (LC MS/MS) is a rapid and sensitive analytical method used for the detection of mycotoxins in vegetal products (cereals and cereal-based products). Here, we report the validation parameters of the LC MS/MS method, for the simultaneous quantitative assay of deoxynivalenol, zearalenone, T-2 toxin and HT-2 toxin accordingly to European Legislations concerning the maximal permitted levels and detection methods for mycotoxins.

Keywords: liquid chromatography; mass spectrometry; deoxynivalenol; zearalenone; T-2 toxin and HT-2 toxin

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Introduction

Mycotoxins are secondary metabolites produced by microfungi that are capable of causing disease and death in animals and humans. The occurrence of mycotoxins in food and feed chain consequently led to the need of their monitoring due to their impact not only on the human health, but also on the industry causing economic loses coming from the negative effects on national and international trade in livestock and livestock products (Cuciureanu, 2008).

Despite of the great efforts made to control the fungal contamination, the toxigenic fungi are ubiquitous in the environment, being frequently met as contaminants of cereals, nuts, fruits and other crops (Bennet and Klich, 2003). From the very large number of mycotoxins, only few are of food safety and health status concern, being constantly produced by *Aspergillus, Fusarium* and *Penicillium* species (Murphy et al., 2006).

Trichothecenes are a large family of chemically related mycotoxins (approximately 148 toxins) produced by various fungal species such as *Fusarium, Trichoderma, Cephalosporium, Myrothecium, Stachybotrys*, which develop in cereal grains and can result in the contamination of the harvested grain, feed and food products thereof (Mihăescu et al., 2005). *Fusarium* species, grow and invade crops under moist cool conditions. Although *F. trinctum* could grow on different plants, the highest amount of mycotoxins is produced on maize, because this plant offers to the fungus ideal conditions for multiplication (Christensen and Nelson, 1976).

Trichothecenes mycotoxins may affect animal and human health as they exhibit an immunosuppressive effect following nucleic acids and proteins synthesis, as well as the inhibition of the mitochondrial function (Rocha et al., 2005). The most common toxins that produce damages on human and animal health are deoxynivalenol, T-2 toxin and HT-2 toxin (Visconti,

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2001). They could cause neural disturbance, haemorrhage, skin irritation, vomiting, diarrhoea and weight loss.

The most critical toxin from the group is T-2 toxin, which differs from HT-2 toxin because of the acetyl group bound in C4 position. Both toxins could be found in the same contaminated products. T-2 toxin could be toxic after inhalation, but its toxicity and lethality are higher in contact with the skin, reason for which it is considered a vesicant agent. After inhalation, the toxin produce nose secretion, itching and sneezing, coughing, breathing difficulties, pulmonary haemorrhage. Severe poisoning produces spasmodic movements, collapse, shock and death (Coman and Popescu, 1985).

Deoxynivalenol (DON) is one of the most common mycotoxins found in grains. DON is produce by *Fusarium* specie, such as *F. graminearum, F. solani, F. oxisporum* (Desjardins et al*.,* 1993). DON toxin is most common, due to its very good stability during storage and food processing and high resitance to degradation during the processing of food, but has lowest toxicity. The most obvious effect is the food refusing. For this reason, deoxynivalenol is sometimes called vomitoxin or food refusal factor (Bennett and Klich, 2003). The toxin has been associated with the acute gastrointestinal syndrome in humans, the major mechanism of action being the inhibition of protein synthesis.

Zearalenone (ZEA) is a mycotoxin produced by different species of *Fusarium,* such as *F. graminearum, F. Nivale* and *F. roseum*. These species colonize particularly cereals (maize, wheat, rice and barley), both during growing stage and post-harvesting, in moisture conditions. Much of the early literature uses zearalenone and F-2 as synonyms. Their chemical structure related to the steroid hormones leads to high binding affinity for the oestrogen hormone receptor, increasing the negative impact on the health status by the anabolic estrogenic effects, depending on dose and host reactivity. ZEA in small amounts produces an anabolic effect, however, its major effect is due to the oestrogen-like activity causing precocious female sexual maturity, infertility, abortion and negative effects on spermatogenesis (Crivineanu et al*.,* 1996).

The World and European Commissions are strongly recommending the prevention and reducing the mycotoxins level in food and feed.

The purpose of this study was the validation of liquid chromatography-tandem mass spectrometry (LC MS/MS) method parameters, accordingly to European Legislations concerning the maximal permitted levels and detection methods for mycotoxins.

Materials and Methods

The samples were weighted (25g ± 0.1), and after adding 100 ml methanol (70 %), they were shaked on vortex for 15 minutes and then filtered. From the filtered solution, 2 ml were mixed with 48 ml phosphate buffer solution (PBS), and 20 ml from the previously obtained solution were transferred into the immunoaffinity column. Before transfer, the affinity column was prepared by connecting it to the vaccum and adding 20 ml from the filtered solution with a debit transfer of 1 drop/minute, then the column was washed with 20 ml of deionised water and dried under air conditions for 30 seconds. The mycotoxins were eluted with 1 ml of methanol (100%), then evaporated under gentle stream of nitrogen and reconstituted in 0.5 ml methanol. Finally, the obtained solution was shaken for 30 s on vortex and injected in LC-MS/MS.

Mycotoxins standard preparation

The calibration curve was performed using a dilution scheme from the concentrated standards of each mycotoxin DON, ZEA, T-2 and HT-2 (table 1). The working concentrations range varied depending on the level of interest. Linearity of the LC-MS/MS system was evaluated by assessing the signal responses of the target analyte from standard solution. In almost all cases, the coefficient of determination (r2) was higher than 0.99.

Table 1: Calibration curve

Level	Concentration of the mycotoxins mixture (ng/ml)			
	DON	ZEA	T2	HT2
1	50	25	10	10
2	100	50	20	20
3	300	100	40	40
4	500	200	60	60
5	1000	300	80	80
6	2000	400	100	100

Table 2: Mobile phase gradient

Rate (timp)	Mobile phase A %	Mobile phase B %	Flow (ml/min)
00.00 min.	100	0	0.3
25.00 min.	15	85	0.3
25.01 min.	100	0	0.3
35.00 min.	100	0	0.3

Chromatography conditions

Mobile phase A: 100 ml methanol + 10 ml acetic acid + 890 ml distilled water + 0.384 g ammonium acetate 5mM; Mobile phase B: 990 ml methanol + 10 ml acetic acid + 0.384 g ammonium acetate 5mM;

LC-MS/MS Varian model 320 working conditions: column Pursuit XRs C18 150 x 3.00 mm, column temperature 40^{0}C, detector 1400V, shield 600 V, needle 4500 V, drying gas 30 psi, nebulizer gas 50 psi, ionisation gas 1.8 Torr, injection volume 15 µl, type of ionization ESI in negative mode for DON and ZEA, for T-2 and HT-2 a positive mode was used. The mobile phase gradient is presented in table 2.

Results and Discussion

The liquid chromatography coupled with MS/MS is one of the most used methods for the assay of mycotoxins (zearalenone, deoxinivalenol, T-2 toxin and HT-2 toxin) from the vegetable products such as unprocessed cereals, breakfast cereals, wheat flour, the concentration treshold being between 10 µg/kg and 2000 µg/kg. The procedure requires the optimization of the equipment working parameters, samples preparation and analysis of the results. The method presented below is internally validated in the laboratory considering the European Union (EU) legislation, in terms of maximum residues level and quality control assurance, and other mycotoxins analysis methods, revised by other authors (Krska et al. 2005; van Osenbruggen and Patterson, 2005; Pascale and Visconti, 2007; Visconti et al. 2007).

The LC MS/MS method is based on mycotoxins extraction from the vegetable products using different reagents (acetonitrile, methanol, water, buttered), clean-up step using a specific immunoaffinity column follow up by separation process of (DON, ZEA, T-2 and HT-2) using a reversed chromatography column.

The analytical limit of detection (LOD) for each mycotoxin was determined as the minimum concentration of the analyte providing a signal-to-noise ratio (S/N) of less than three and a limit of quantification (LOQ) of 10. The recovery studies were performed on different levels for mycotoxins. The results of recovery were in range of 72-122%. At the same concentrations, the experiment was repeatable (n=5), with relative standard deviations (RSD) ranging from 1.6-12.4 (Table 3). The minimal detected levels were in accordance with the European Commision maximum permitted levels for DON and ZEA under Regulation (CE) No 1881/2006 and Regulation CE No 1126/2007 (i.e wheat 1750 µg/kg DON and 100 µg/kg ZEA), while T-2 and HT-2 toxins are covered by Recommendation No 165/2013 for cereals.

Selected reaction monitoring experiments were carried out to obtain the maximum sensitivity for the detection of the target molecules (Fig. 1). The quantitative analysis of each mycotoxin was carried out using the selected ion monitoring mode of each base ion peak at *m/z* 354.9 (DON), 317 (ZEA) in the negative mode and *m/z* 484.1 (T-2), 442.1 (HT-2) in the positive mode, along with retention time matching (table 4).

Conclusions

The liquid chromatography-tandem mass spectrometry (LC MS/MS) is a sensitive analytical method used for the simultaneous quantitative assay of deoxynivalenol, zearalenone, T-2 toxin and HT-2 toxin in vegetal products. The method validation parameters are in accordance with the EU legislation for the maximum permited limits (Commission Regulation

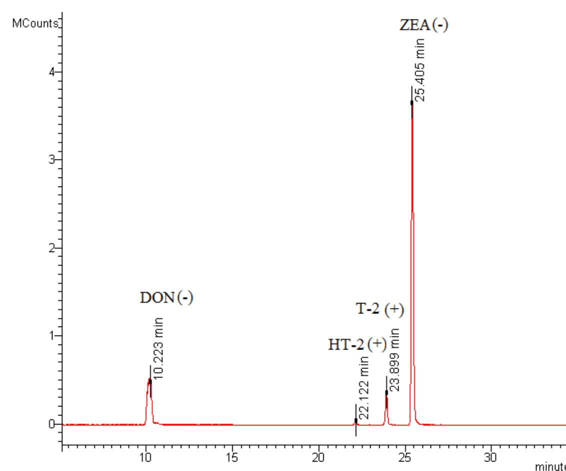

Fig. 1: Chromatogram of spike samples DON (100 µg/kg), ZEA (50 µg/kg), T-2 (20 µg/kg) and HT-2 (20 µg/kg).

Table 3: Recovery rate average, limit of detection, limit of quantification

Mycotoxins	Spiking level (µg/kg)	Average recovery (%)	RSD (%)	LOD (µg/kg)	LOQ (µg/kg)
DON	1250	72.5	12.4	50	100
	100	108.45	3.3		
ZEA	100	100	6.2	25	50
	50	81.60	2.8		
T-2	30	122.32	4.0	10	20
	40	82.75	4.3		
HT-2	20	72.1	1.6	10	20
	30	76.3	4.4		

Table 4: MS/MS Parameters

Mycotoxins	Retention time (min.)	Ion Polarity	Q1(m/z)	Q3 (m/z)	Capilary (V)	Colision energy (V)	Ionization mode
DON	10.22	[DON+CH3COO]⁻	354.9	294.7 quantification ion	45	21	Negative
				137.8 qualification ion		9	
ZEA	22.12	[ZEA-H]⁻	317	130.4 quantification ion	90	30	Negative
				186.6 qualification ion		24	
T-2	23.89	[T-2+NH4]⁺	484.1	185.0 quantification ion	50	13.5	Positive
				245.0 qualification ion		13	
HT-2	25.40	[HT-2+NH4]⁺	442.1	215.0 quantification ion	35	12.5	Positive
				263.0 qualification ion		12.5	

Q1=quadrupole 1 ; Q3=quadrupole 3

(EC) No 1881/2006 and Commission Recomandation No 165/2013) and quality control assurance of determination (Commission Regulation (EC) No 401/2006). The LC-MS/MS method represents a rapid, effective and sensitive method for the concomitant quantitative assay of mycotoxins in cereals.

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Commission Regulation (EC) No 401/2006 laying down the methods of sampling and analysis for the official control of the level of mycotoxins in food stuffs.

Commission Recomandation 165/20013 on the presence of T-2 and HT-2 toxins in cereals and cereal products.