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Simultaneous determination of multi – component mycotoxin in feeds by liquid chromatography – tandem mass spectrometry

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Abstract

A multiresidue method for determination and quantification of *Fusarium* mycotoxins: deoxynivalenol, zearalenone, T-2 toxin, HT-2 toxin, and metabolite of *Aspergillus* and *Penicillium* species - ochratoxin A in feeds was described. The method was based on the simultaneous extraction of selected mycotoxins from matrix, followed by liquid chromatography coupled with tandem mass spectrometry using a hybrid triple quadrupole – linear ion trap mass spectrometer with the multiple reaction monitoring in both positive- and negative-ion modes. The method was validated in accordance with the Commision Decision 2002/657/EC requirements. The mean recoveries of mycotoxins from spiked feed samples ranged from 74.6% to 113.9%, whereas limit of detection and quantification ranged from 0.72 to 12.4 µg/kg and 1.86 to 31.7 µg/kg, respectively.

Key words: feeds, mycotoxins, chromatography, mass spectrometry, validation.

Introduction

Mycotoxins are toxic secondary metabolites produced by filamentous fungi, as *e.g. Fusarium*, *Aspergillus,* and *Penicillium* species, growing under a wide range of climatic conditions on agricultural commodities (grains, spices, fruits, coffee, nuts, etc.) in the field and during storage. Their presence in food and feed has been identified as a potential threat to the human and animals' health, which can be caused by direct contamination of vegetable or their products, or „transfer" of mycotoxins and their metabolites in animal tissues, milk, and eggs (15, 23). Agricultural products may be contaminated at any stage, from the development of plants in the field (*Fusarium* sp. and *Aspergillus* sp.), during harvest and processing (*Penicillium* species and *Aspergillus* sp.), storage, and transport. Mycotoxins are not destroyed in the course of processing and storage of feed, and can be cumulated. Mycotoxins - deoxynivalenol (DON), zearanelone (ZEN), T-2 toxin, HT-2 toxin, and ochratoxin A (OTA), in temperate climates have a particular impact on the health of humans and animals (2). These toxins should be of a great interest because of an increasing evidence of their involvement in human and animal diseases (3, 10, 23). In 1993, the International Agency for Research on Cancer (IARC) has added OTA to the register as a possible carcinogen, and DON, ZEN, and T-2 and HT-2 toxins, which are impossible to classify as non-carcinogenic for humans (11). These compounds can cause the refusal of food, anaemia, haemorrhages, immunosuppression, neurotoxic effects, nephrotoxicity, hepatotoxicity, damage of the bone marrow, and a possible carcinogenesis (14).

Contamination of raw materials, food, and feed by mycotoxins is a major health and economic problem. The awareness of the threat of mold, and thus the emergence of mycotoxins was reflected in the global, European, and Polish legislation. Currently, in Poland contamination of foods, including grains and seed crops, with mycotoxins is regulated by Commission Regulation (EU) No. 1881/2006 setting maximum levels for certain contaminants in foodstuffs (5). These compounds penetrate the human body, not only from contaminated food products of plant origin, but also by contaminated animal products (meat, milk, and eggs) deriving from animals fed contaminated feed. The health effects of exposure to mycotoxins - called

mycotoxicosis, are of different course, from acute to chronic poisoning arising from ingestion of small doses for a long period of time. It can be agreed that mycotoxins as substances are potentially carcinogenic to humans, which cannot be completely eliminated, should be limited to the lowest possible levels. Determining these limits requires a consideration of multiple factors including climatic conditions, feeding habits in different countries, level of toxicological data availability, risk assessment, appropriate analytical methods, and proper sampling procedures (4–8).

Several analytical procedures have been developed for the determination of *Fusarium* mycotoxin compounds and OTA in feed. Analysis of DON, ZEN, OTA, T-2, and HT-2 toxins from feed traditionally applies thin – layer chromatography (TLC), liquid chromatography (LC) coupled with ultraviolet (UV), photo diode array (PDA), fluorescence detectors (FLD) or mass spectrometry (MS), and gas chromatography (GC) coupled with electron capture (ECD), flame ionisation (FID), or MS detectors (1, 13, 16, 17, 19–22, 24, 25).

The aim of the study was to develop a LC–MS/MS method for simultaneous determination of several mycotoxins. Previously, the Institute for Reference Materials and Measurements in Geel, Belgium, applied and disseminated the procedure for the determination of DON, ZEN, T-2, HT-2 in feed (12). Our method has been based on this procedure, but we added OTA as the most important mycotoxin under climatic conditions in Poland (2, 18). After the modification and validation according to the European Decision 2002/657/EC (9), the procedure can be used for the official control of veterinary and diagnostic tests.

The LC system was coupled to a triple – quadrupole mass spectrometer equipped with electrospray ionisation (ESI) probe. ESI-MS/MS performed in the multiple reaction-monitoring (MRM) mode with positive and negative polarities. The selected precursor ions of the analytes were fragmented to their product ions. The two most intensive product ions per analyte were chosen for quantitative and confirmation purposes.

Material and Methods

Reagents and chemicals. Solvents: methanol (analytical grade), ethyl acetate, and anhydrous sodium sulphate were obtained from POCh (Poland); methanol (analytical HPLC, resi grade) and acetonitrile were purchased from J.T. Baker (the Netherlands); formic acid was obtained from Sigma-Aldrich (Germany); purified water was obtained with a Milli-Q apparatus (USA). Standards of DON, ZEN, T-2, HT-2, and OTA, internal standards of DON-$^{13}C_{15,}$ ZEN-$^{13}C_{18}$, T-2-$^{13}C_{24,}$ HT-2-$^{13}C_{22}$, and OTA-$^{13}C_{20,}$ were purchased from Sigma-Aldrich (Germany). Structure of molecules of mycotoxins are presented in Fig. 1. All standards were kept according to the recommendations of the certificates. Primary standard stock solution and working solutions were prepared in acetonitrile and mobile phase at concentration of 1000 µg/mL to 1 ng/mL at 2–8°C.

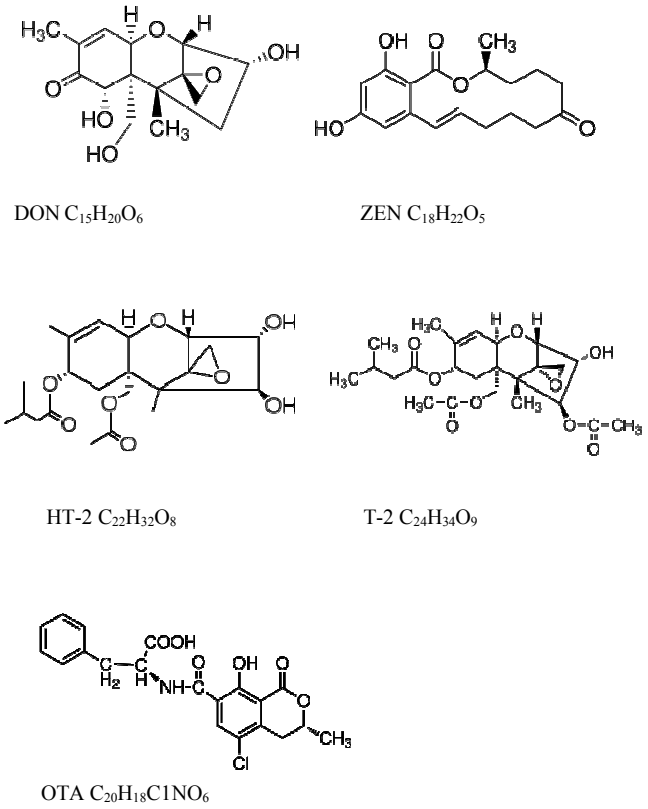


Fig. 1. Structure of molecules of DON, ZEN, HT-2, T-2, and OTA (21)

Sample preparation and extraction. Each sample was ground in a laboratory mill (Glen Mills Inc., USA), sieved (dimension of mesh 1 × 1 mm), mixed for the achievement of homogeneity, and stored at ≤-18ºC until analysis. Four millilitres of water and 8 mL of ethyl acetate were added to 1 g of the samples (working stock solution was added to control samples) and shaked for 30 min in orbital shaker. Then, 4 g of sodium sulfate were added to each sample and the samples were homogenised with Vortex mixer. The mixtures were centrifuged at 3500 rpm for 5 min at temperature about 10°C. Afterwards, 0.5 mL of supernatants was transferred to clean tubes. At this point, solution with internal standards was spiked to all samples. The samples were evaporated until dry using nitrogen at 60°C. The dry residues were dissolved in 0.5 mL of 0.1% formic acid in methanol and injected into the LC-MS/MS.

Quantification of DON, ZEN, T-2, HT-2, and OTA. LOD and LOQ were obtained by the analysis of the background noise of 20 different blank samples. The results were checked by analysis of 10 samples

spiked on LOD and LOQ levels. The feed samples were spiked with DON, ZEN, T-2, HT-2, and OTA at the levels of 576–2560 µg/kg, 54–240 µg/kg, 54–240 µg/kg, 90–400 µg/kg, and 2.5–7.5 µg/kg, respectively, and processed through the extraction procedure. The internal standards were used for quantitation after spiking with DON-$^{13}C_{15}$, ZEN-$^{13}C_{18}$, T-2-$^{13}C_{24}$, HT-2-$^{13}C_{22}$, and OTA-$^{13}C_{20}$. The recoveries of mycotoxins were evaluated by comparing with the concentrations found in the standard solutions. The precision of the method was measured using the same samples.

LC-MS/MS analysis. Agilent Series 1200 HPLC system (Agilent Technologies, Germany) with QTRAP®5500 triple quadrupole mass spectrometer (AB SCIEX, Canada) and Analyst 1.5.2 software were used for the analysis. The mass spectrometer was operated in electrospray positive (ESI+) and negative (ESI–) ionisation mode, and two multiple reaction monitoring (MRM) transitions for each analyte were monitored. The following mass spectrometer parameters were used: resolution Q1 and Q3 - unit, nebuliser gas - 35 psi, curtain gas - 20 psi, collision gas - average level, ion spray voltage - 4500V/4500V, temperature - 350°C.

The chromatographic separation of mycotoxins was performed with the Supelco Ascentis® Express C18 column (75 × 2.1 mm; particle size 2.7 µm fused – core) using 0.3 mL/min of constant flow. The mobile phase for analysis consisted of 0.1% formic acid in water – 0.1% formic acid in methanol in gradient elution (Table 1). The injection volume was 10 µL.

Table 1. Programme of gradient mobile phase

Time (min)	Formic acid in water (%)	Formic acid inmethanol (%)
0.0	95	5
2.0	30	70
6.0	20	80
6.5	5	95
8.0	5	95
10.0	95	5
15.0	95	5

Results

During the development of the method of detection, the following conditions of MS/MS were optimised for each analyte: fragmentation reactions, ionisation mode, collision energy (CE), the voltage at the output of the collision chamber (CXP), cone voltage (DP), and dwell time (Table 2).

The method was validated with accordance to the Commision Decision 2002/657/EC requirements (9). The summary of the validation results of mycotoxins in feed samples is presented in Table 3.

The results show a good recovery, precision (repeatability and within-laboratory reproducibility), limit of detection, and limit of quantification. The calibration curves for spiked samples of mycotoxins were prepared, and the parameters of linear regression were estimated. The optimal internal standard in MS had the same chemical structure as the analysed compound, but a different *m/z* ratio. For proposed analytical and detection conditions, correct chromatographic separation of DON, ZEN, T-2, HT-2, and OTA was obtained (Fig. 2).

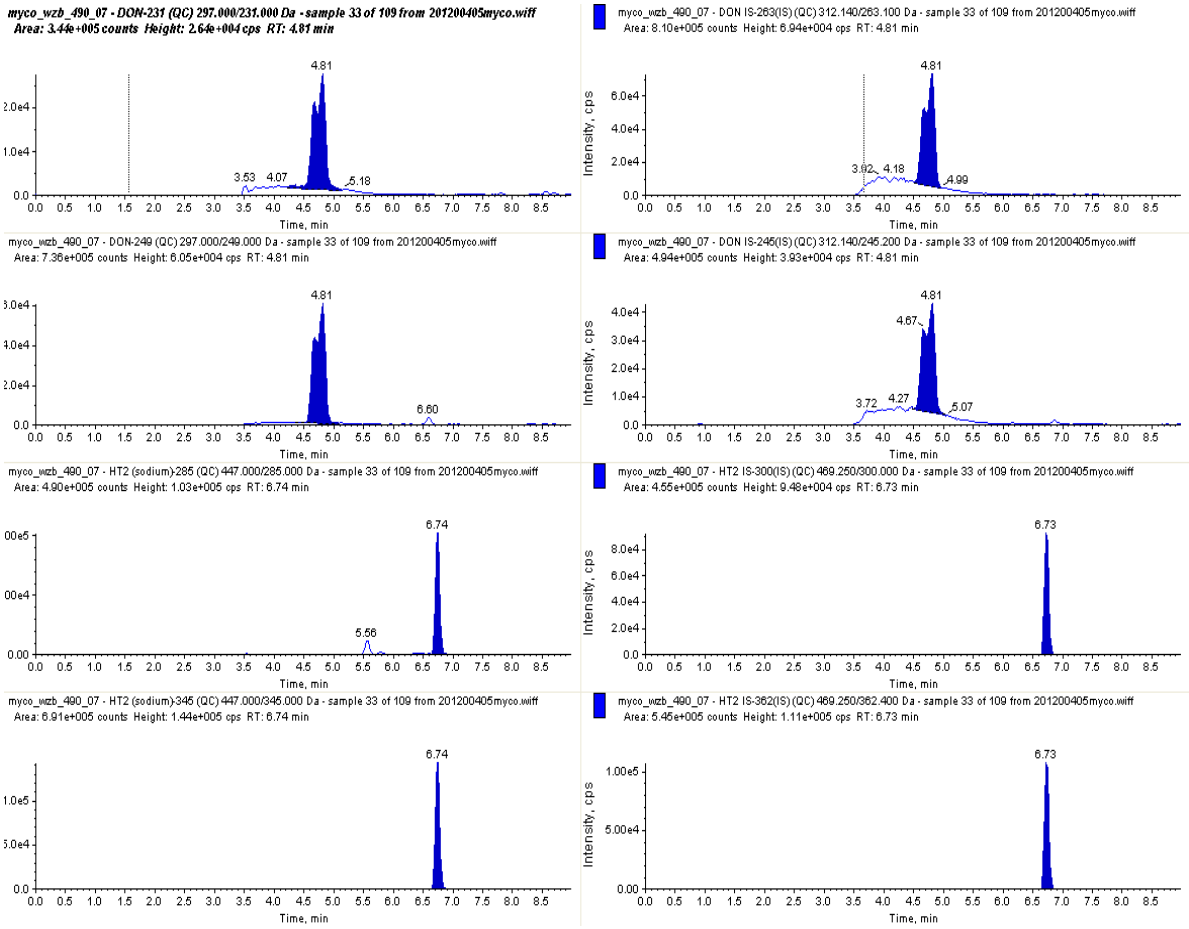
Table 2. MS/MS parameters on the parent and quantitative daughter ion (*m/z*) of mycotoxins

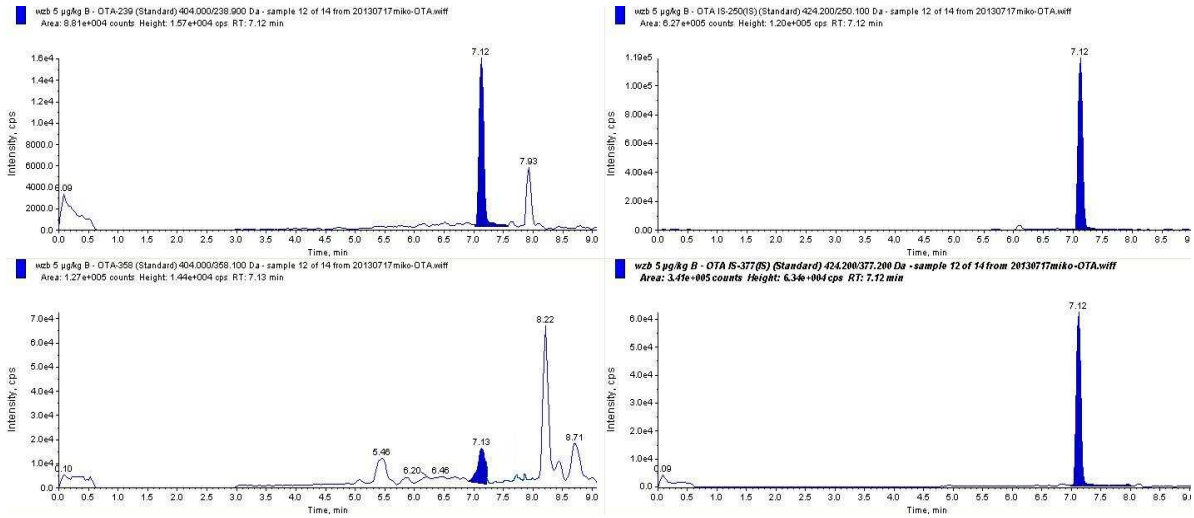
Analyte	Transitions	Ionisation mode	CE (V)	CXP (V)	DP	Dwell time (ms)
Zearalenone	317 → 131	ESI–	38	15	270	70
	317 → 175		32	9	270	70
Zearalenone (IS) – $^{13}C_{18}$	335 → 185		34	9	135	70
	335 → 290		30	11	135	70
Deoxynivalenol	297 → 231	ESI+	19	22	126	70
	297 → 249		15	18	96	70
Deoxynivalenol (IS) – $^{13}C_{15}$	312 → 263		15	22	86	70
	312 → 245		19	12	86	70
T-2 Toxin	489 → 245	ESI+	37	24	58	70
	489 → 327		31	14	58	70
T-2 Toxin (IS) – $^{13}C_{24}$	513 → 260		39	24	81	70
	513 → 344		33	32	81	70
HT-2 Toxin	447 → 285	ESI+	29	16	90	70
	447 → 345		25	22	90	70
HT-2 Toxin (IS) – $^{13}C_{22}$	469 → 362		23	24	146	70
	469 → 300		29	14	146	70
Ochratoxin A	404 → 239	ESI+	33	16	70	70
	404 → 358		21	14	70	70
Ochratoxin A (IS) – $^{13}C_{20}$	424 → 250		35	14	101	70
	424 → 377		21	14	101	70

IS – internal standard, CE – collision energy, CXP – voltage at the output of the collision chamber, DP – cone voltage

Table 3. Summary of results of method validation

Parameters	Deoxynivalenol			Zearalenone			T-2 Toxin			HT-2 Toxin			Ochratoxin A		
Linear regression equation, (y = ax + b)	y = 0.0791x + 60.55			y = 0.9963x + 4.5641			y = 0.9499x + 0.9069			y = 0.79x + 11.2857			y = 0.0264x + 0.012		
Correlation coefficient of calibration curve (R^2)	1.0000			0.9994			0.9999			0.9999			0.9966		
Linearity (working range), µg/kg	80–2560			7.5–240			8–240			13–400			1.25–15		
Limit of detection, µg/kg (LOD)	12.4			8.7			3.8			3.32			0.72		
Limit of quantification, µg/kg (LOQ)	31.7			15.0			6.1			8.21			1.86		
Level of spiked samples feed, µg/kg	576	1568	2560	54	147	240	54	147	240	90	245	400	2.5	5.0	7.5
Repeatability, CV%	4.5	5.2	1.5	4.8	3.4	2.4	2.8	1.8	1.3	3.2	2.3	1.4	2.7	3.8	4.7
Within-laboratory reproducibility,CV%	6.2	6.4	7.9	5.7	9.3	9.8	14.1	13.8	11.3	6.3	11.7	7.6	8.4	3.0	3.7
Recovery, %	98	91	82	100	113	103	89	85	85	84	79	74	100	100	101





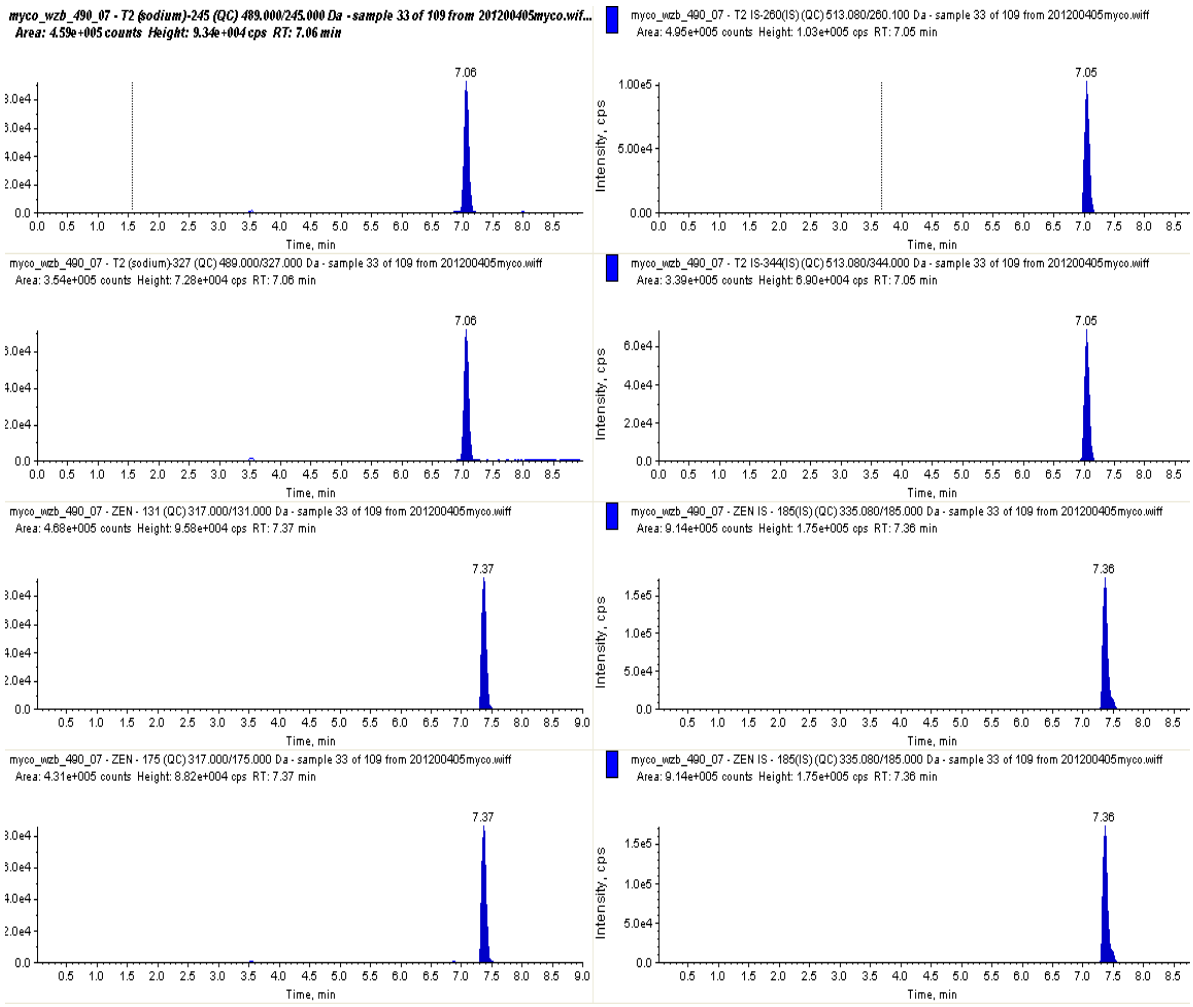


Fig. 2. LC-MS/MS chromatograms of spiked samples of DON at 1568 µg/kg, HT-2 at 245 µg/kg, T-2 at 147 µg/kg, ZEN at 147 µg/kg and OTA at 5 µg/kg

Discussion

The presence of contaminants in food and feed is one of the basic criteria for assessing the safety of food products. The studies on mycotoxins are intended not only to establish the extent of their toxicity, but also the impact on human health, and are moving towards the development of effective methods for the determination of these compounds. The detection of several mycotoxins in a single course of analysis is particularly important because of the possibility of simultaneous accumulation of these compounds in the feed (18).

A rapid quantitative method for the simultaneous determination of the major mycotoxins: DON, ZEN, T-2, HT-2, and OTA in feed using LC-MS/MS detection, was developed on the basis of the method described by the Institute for Reference Materials and Measurements in Geel (12). In our study, the number of analytes was increased by adding OTA. During testing, it was noticed that the use of a solution of 0.1% formic acid in methanol to dissolve the dry residue after evaporation under nitrogen, reduces the contamination of the sample and the filtration step is not required. Additionally, low temperature during centrifugation of extracts enables to obtain better purification and recoveries. In this research, simple extraction combined with fast LC separation greatly reduced the analysis time and made this method suitable for routine use. Sample preparation is often the most critical part of a multiresidue method, due to the different properties of mycotoxins that have to be isolated simultaneously from the feed. Mycotoxins are compounds of low molecular weight, weakly polar, heat stable, and do not decompose during pasteurisation, even at higher temperatures. Generally, organic solvents were used for extraction in the quantitative procedures of mycotoxin analysis (22). In many studies, the extraction from samples was performed with acetic acid, acetonitrile, methanol, water, and mixture of solvents (1, 13, 16, 17, 19, 20, 22, 24, 25). We used ethyl acetate preceded by wetting the sample with water, and the recoveries were satisfactory (Table 3).

The best chromatograms for all mycotoxins were obtained using a mobile phase consisting of formic acid (0.1%) in water and formic acid (0.1%) in methanol with a gradient elution programme (Table 1).

Mycotoxins have a wide range of polarity strengths; therefore a mobile phase with a variable degree of hydrophobicity over the time course of each analysis was required. Thus, the chromatography was started with a mobile phase having a low organic solvent content for the elution of the more polar mycotoxins, and the organic solvent content was gradually increased to elute the less polar mycotoxins within a reasonable time frame. After the extraction with liquid-liquid, clean-up, extract was concentrated and dissolved in 0.1% formic acid in methanol. Then, extracts were injected to the LC-MS/MS instrument. The specificity and sensitivity of the modern mass spectrometric instruments allow for this type of approach, and extensive purification of the final sample and complete chromatographic separation (of both the analytes and the matrix components) are no longer required. This facilitates the overall analysis and decreases its costs. The use of good internal standard is very important in order to obtain a robust and quantitative method in LC-MS/MS.

The use of a triple quadrupole mass spectrometer allows the simultaneous identification of these five mycotoxins and their quantification at different levels. The increased analytical throughput and the determination of different groups of mycotoxins hold great promises for this and other LC-MS/MS analyses in the future. The method is time-saving, cost-efficient, flexible, and new compounds can be added easily. The developed procedure meets the required performance criteria for methods of mycotoxins determination, which was confirmed in proficiency testing programme, organised by the EURL-Geel, Belgium. The developed multimycotoxin method permits simultaneous, simple and rapid determination of several co-existing toxins at relatively low concentration levels, and is ideal for, *e.g.*, screening-type work and can be used for the official control of veterinary and diagnostic tests.

References

1. Berthiller F., Sulyok M., Krska R., Schuhmacher R.: Chromatographic methods for the simultaneous determination of mycotoxins and their conjugates in cereals. Int J Food Microbiol 2007, 119, 33–37.
2. Chełkowski J.: Mikotoksyny i grzyby toksynotwórcze, jako istotny wskaźnik jakości żywności i pasz. Polskie Drobiarstwo 2008, 10, 22–26.
3. Dworecka - Kaszak B.: Mikologia weterynaryjna. Edited by SGGW, Warszawa, 2008.
4. European Commission Regulation No. 576/2006. Off J Eur Commun L 229, 7–9.
5. European Commission Regulation No. 1881/2006. Off J Eur Commun L 364, 15–17.
6. European Commission Regulation No. 401/2006. Off J Eur Commun L 70, 12–34.
7. European Commission Regulation No. 152/2009. Off J Eur Commun L 54, 1–130.
8. European Commission Regulation No. 165/2013. Off J Eur Commun L 91, 9–12.
9. European Commission Regulation of 12 August 2002 No. 657/2002 implementing Council Directive 96/23/EC.
10. Hussein H.S., Brasel J.M.: Toxicity, metabolism and impact of mycotoxins on humans and animals. Toxicology 2001, 167, 101–134.
11. IARC monographs on the evaluation of carcinogenic risks to humans - some traditional herbal medicines, some mycotoxins, naphthalene and styrene 2002, 82, 169–345.
12. Institute for Reference Materials and Measurements, Food Safety and Quality – Geel, Belgium: Method for the simultaneous determination of deoxynivalenol, HT-2 toxin, T-2 toxin and zearalenone in unprocessed cereals and cereal-based compound animal feeds, 2011.
13. Kokkonem Meri K., Jestoi Marika N.: A multi – compound LC-MS/MS method for the screening of mycotoxins in grains. Food Anal Method 2009, 2, 128–140.
14. Kolenda M., Mroczkowski S.: *Fusarium mycotoxins* and methods of assessing the mycotoxicity: a review. J Centr Europ Agric 2013, 1, 169–180.
15. Korbas M., Ławecki T.: The possibility of reducing *Fusarium* blight in Poland and in the European Union. Postępy w Ochronie Roślin 2003, 41, 200–207.
16. Monbaliu S., Van Poucke C., Detavernier C., Dumoulin F., Van De Velde M., Schoeters E., Van Dyck S., Averkieva O., Van Peteghem C., De Saeger S.: Occurrence of mycotoxins in feed as analyzed by a multi-mycotoxin LC-MS/MS method. J Agric Food Chem 2010, 58, 66–71.
17. Oueslati S., Romero-González R., Lasram S., Frenich A.G., Vidal J.L.: Multi-mycotoxin determination in cereals and derived products marketed in Tunisia using ultra-high performance liquid chromatography coupled to triple quadrupole mass spectrometry. Food Chem Toxicol 2012, 50, 2376–2381.
18. Piotrowska M.: The use of microorganisms for the removal of mycotoxins in food and feed. Post Mikrobiol 2012, 51, 109–119.
19. Soleimany F., Jinap S., Abas F.: Determination of mycotoxins in cereals by liquid chromatography tandem mass spectrometry. Food Chem 2012, 130, 1055–1060.
20. Soleimany F., Jinap S., Faridah A., Khatib A.: A UPLC – MS/MS for simultaneous determination of aflatoxins, ochratoxin A, zearalenone, DON, fumonisins, T-2 toxin and HT-2 toxin, in cereals. Food Control 2012, 25, 647–653.
21. Song S., Ediage E.N., Wu A., De Saeger S.: Development and application of salting-out assisted liquid/liquid extraction for multi-mycotoxin biomarkers analysis in pig urine with high performance liquid chromatography/tandem mass spectrometry. J Chromatogr A 2013, 1292, 111–120.
22. Sulyok M., Krska R., Schuhmacher R.: Application of a liquid chromatography – tandem mass spectrometric method to multi – mycotoxin determination in raw cereals and evaluation of matrix effects. Food Additives and Contaminants 2007, 24(10), 1184–1195.
23. Sweeney M., Dobson A.: Mycotoxin production by *Aspergillus, Fusarium* and *Penicillium* species. Int. J. Food Microbiol 1998, 43, 141–158.
24. Tanaka H., Takino M., Sugita-Konishi Y., Tanaka T.: Development of a liquid chromatography/time-of-flight mass spectrometric method for the simultaneous determination of trichothecenes, zearalenone and aflatoxins in foodstuffs. Rapid Commun Mass Spectrom 2006, 20, 1422–1428.
25. Zöllner P., Mayer-Helm B.: Trace mycotoxin analysis in complex biological and food matrices by liquid chromatography-atmospheric pressure ionization mass spectrometry. J Chromatogr A 2006, 1136, 123–169.