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METHOD OF LIQUID CHROMATOGRAPHY – TANDEM MASS-SPECTROMETRY FOR ENNIATINS A, A1, B, B1 AND BEAUVERICIN DETERMINATION IN WINTER WHEAT

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Abstract

Objective: *the aim of this study is to develop and validate a method of liquid chromatography – tandem mass-spectrometry for determination Fusarium mycotoxins namely eniatins A, A1, B, B1 and beauvericin.* ***Methods:*** *this article considers an analytical method based on high-performance liquid chromatography coupled with tandem mass-spectrometry for quick separation, identification, and quantification of mycotoxins in one sample.* ***Results:*** *it is proved that proposed method is accurate, selective, and rapid for identification and quantification of law-molecular weight compound accumulated in grain in small amount.* ***Discussion:*** *the effectiveness of liquid chromatography – tandem mass-spectrometry for determination of low concentrated mycotoxins in samples is confirmed. The validation ranges for analysts are compared to similar aimed researches of past years.*

Keywords: beauvericin; enniatins; *Fusarium spp.*; liquid chromatography – tandem mas-spectrometry; mycotoxins.

1. Introduction

On the territory of Ukraine winter wheat (*Triticum spp.*) ranks first in sown areas (6.1 million Ha) [1] and is the main food crop among the most important cereals. Currently, most of wheat production is used as feed: the best quality wheat is utilized for production of bread and bakery products. The quality of grain depends on the favorable chemical composition. *Fusarium, Alternaria, Aspergillus* species are the most considerable fungal pathogens attacking cereals [2]. Mycotoxins, secondary metabolites of these molds are low-molecular-weight toxic chemical compounds that settle crops, in the field or during storage, and are capable of causing disease and death in humans and other animals. The diseases caused by affecting of mycotoxins are kidney damage, gastrointestinal disturbances, reproductive disorders or suppression of the immune system [3]. Trichothecenes, zearalenone, fumonisins are the most prevalent mycotoxins produced by *Fusarium* species. However, beauvericin and enniatins can dangerous affect living beings as mycotoxins listed above, them are no routinely determined.

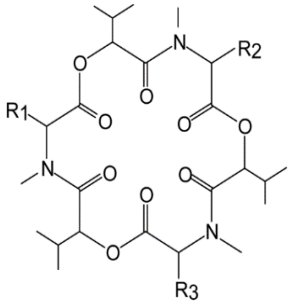
Enniatins and beauvericin are cyclic hexadepsipeptides consisting of alternating units of D-α-hydroxyisovaleryl and three N-methyl-L-amino acid residues (Fig. 1) [4]. Enniatins exert antibiotic activities against various bacteria, exhibit immunomodulatory properties, and are potent inhibitors of mammalian cholesterol acyl transferase [5]. Beauvericin shows antibiotic activity against several bacteria, has moderate insecticidal properties, causes cytolysis and induces programmed cell death [6]. Both enniatins and beauvericin possess ionophore properties: they change pH and physiological ionic balance.

Mycotoxins can pass from contaminated grain to cereal products. Therefore, their concentrations are regulated.

With developing of liquid chromatography – tandem mass-spectrometry (LC-MS/MS), determination of low-molecular weight compound become faster and more selective. This accurate and

sensitive analytical method based on high-performance liquid chromatography (HPLC) and mass-spectrometry (MS) [7-8] is applied for quick determination of enniatins and beauvericin in one sample.

This paper reports the development and validation of a LC-MS/MS method for the determination of enniatins A, A1, B, B1 and beauvericin.



Compound	R_1	R_2	R_3
Enniatin A	$CH(CH_3)C_2H_5$	$CH(CH_3)C_2H_5$	$CH(CH_3)C_2H_5$
Enniatin A1	$CH(CH_3)C_2H_5$	$CH(CH_3)C_2H_5$	$CH(CH_3)_2$
Enniatin B	$CH(CH_3)_2$	$CH(CH_3)_2$	$CH(CH_3)_2$
Enniatin B1	$CH(CH_3)_2$	$CH(CH_3)_2$	$CH(CH_3)C_2H_5$
Beauvericin	$CH_2C_6H_5$	$CH_2C_6H_5$	$CH_2C_6H_5$

Fig. 1. Simplified structure of enniatins and beauvericin

2. Analysis of the latest research and publications

Scientists devoting their research efforts to the Fusarium mycotoxins field interest the recent developed method for toxins identification and quantification from different plant matrixes (wheat, barley, corn etc). Method of LC-MS/MS has began to be developed and used in agriculture for enniatins and beauvericin determination since first years of new millennium [8]. Current investigations directed on mycotoxins identification by LC-MS/MS that are reported for general overview in scientific database PubMed are counted 206 papers in 2017 [9].

3. Materials and Methods

Materials.

The subjects of this research are mycotoxins enniatins A, A1, B, B1 and beauvericin, which are determined from flour of winter wheat *Triticum aestivum* and *Triticum turgidum*. Wheat grains are milled on Retsch ZM 100 laboratory mill.

Enniatins and beauvericin were obtained from Sigma. Stock solutions were prepared by dissolution of crystalline material in methanol (0,025-0,4 mg/ml).

Following reagents and solutions for the process of samples preparation were used: 0,1% solution of formic acid (HCOOH); acetonitrile (CH_3CN); $MgSO_4$; NaCl.

For the analytical LC-MS/MS method following reagents were used: methanol (CH_3OH); 0,1% solution of formic acid (HCOOH); ammonium formate ($HCOONH_4$).

Deionized water was prepared using the Milli-Q system.

Preparation of samples.

Samples for LC-MS/MS were prepared using the modified QuEChERS method. The mixture of 10 ml of 0,1% HCOOH and 10 ml of CH_3CN was added to 5g of homogenized sample. The mixture is stirred at 250 rpm during 20 min. Then 4g of $MgSO_4$ and 1g of NaCl were added, and mixture was centrifuged at 5000 rpm during 5 min. 0,5 ml of supernatant was diluted with 0,5 ml of water. Sample was filtered through 0,2 mcm nylon filter.

Analysis of mycotoxins by LC-MS/MS

An Acquity instrument liquid chromatograph combined with a triple quadrupole XevoTQMS (Waters) mass spectrometer were used to identify and quantify enniatins and beauvericin. Chromatographic separation was carried out using a column C18 (50 x 2 mm, 1.7 μm) of Phenomenex at a temperature of 40 ° C.

The mobile phase for the chromatograph contains 0,1% formic acid, 1 mM ammonium formate in methanol.

Conditions for chromatography are: flow rate was 0.3 ml/min, and injection volume 2.5 μl. Conditions for mass-spectrometer were: the electrospray ionization (ESI) interface was used in positive ion mode; source temperature – 150° C; desolvation temperature – 450 ° C, cone nitrogen gas flow – 15 l/h, desolvation gas flow – 700 l/h, capillary voltage – 3 kV.

Information about mycotoxins is obtained and analyzed using the Single Reaction Monitoring (SRM) mode. The precursor and fragments ions for each analyte are given in Table 1.

Data and results are collected and processed by MassLynx software.

4. Results and Discussion

LC-MS/MS analysis

The analysis process of the mycotoxins' content in wheat samples *T. aestivum* and *T. turgidum* is carried out at the important conditions for the seperation of enniatins and beauvericin by liquid chromatography, and their molecular identification by mass-spectrometer.

Accuracy, precision, selectivity, recovery and extended uncertainty of the determination of multiple mycotoxins were verified within the validation process.

Table 1

MS/MS parameters for each analyte

Mycotoxin	Precursor ion	Product ion 1	Collision energy, eV	Product ion 2	Collision energy, eV
Beauvericin	784,4	244	25	262,4	25
Enniatin B	640,4	196,1	25	214,1	25
Enniatin B1	654,4	196,1	30	214,2	30
Enniatin A1	668,5	210,2	27	228,2	27
Enniatin A	682,5	210,3	27	228,3	27

Method validation

The LC-MS/MS method was validated from spike recovery experiments. Fig. 2 shows a LC-MS/MS profile of a spiked wheat sample acquired using SRM mode. The validation ranges for analysts compared with the relative ratio of the fungal mycotoxins in several researches [10-13]. Mycotoxins were quantified using standard curves.

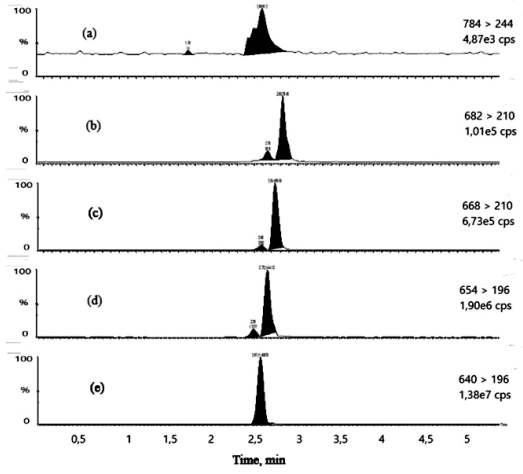


Fig. 2. LC-MS/MS chromatogram of *T. aestivum* spiked at: (a) beauvericin, 0,0004 μg/g; (b) enniatin B, 4,2 μg/g; (c) enniatin B1, 0,6 μg/g; (d) enniatin A1, 0,12 μg/g; (e) enniatin A, 0,02 μg/g

The limits of detection (LoD) and quantification (LoQ) from this experiment were good, and completely contented the same parameters in similar researches [10-13]. The concentrations LoD and LoQ for spiked samples are represented in Table 2.

Occurrence of enniatins and beauvericin in analyzed samples

The results from the presence of four enniatins (A, A1, B and B1) and beauvericin are estimated by the usage of standard curve. All samples of wheat were contaminated with determined mycotoxins in different amount. LC-MS/MS analysis of wheat *T. turgidum* shows that enniatins B and B1 accumulated in the largest amount

Table 2

Validation parameters of LC-MS/MS method

Mycotoxin	LoD, μg/kg	LoQ, μg/kg	RT (min)
Beauvericin	1,5	5,0	2,69
Enniatin B	1,5	5,0	2,94
Enniatin B1	1,5	5,0	2,84
Enniatin A1	1,5	5,0	2,75
Enniatin A	1,5	5,0	2,65

(21,5 μg/g and 3,25 μg/g respectively). Then in amount of 1,43 μg/g enniatin A1 is accumulated. The mass of enniatin A – 0.275 μg/g, and beauvericin – 0.13 μg/g.

5. Conclusion

The liquid chromatography – tandem mass-spectrometry method for the detection of enniatins A, A1, B, B1 and beauvericin was developed and validated. This method was applied to the samples of winter wheat (*T. aestivum* and *T. turgidum*). The detected mycotoxin concentrations did not differ from the values given in the literature in previous years.

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References

[1] Ministry of Agrarian Policy and Food of Ukraine (2017), "A sown area under crop in 2017", available at: www.minagro.gov.ua (Accessed 13 June 2017).

[2] Brakhage A.A. (2013) Regulation of fungal secondary metabolism. *National Review. Microbiology*, No 11, pp. 21–32.

[3] Peraica M., Radić B., Lucić A., Pavlović. M (1999) Toxic effects of mycotoxins in humans. *Bulletin of the World Health Organization*, vol. 77, No. 9, pp. 754-766.

[4] Oueslati, S., Meca, G., Mliki, A., Ghorbel, A., Mañes, J. (2011). Determination of Fusarium mycotoxins enniatins, beauvericin and fusaproliferin in cereals and derived products from Tunisia. *Food Control*, No 22, pp. 1373–1377. doi: 10.1016/j.foodcont.2011.02.015.

[5] Kirst H. A., Yeh W.-K., Zmijewski M.J., Jr (2001) *Enzyme Technologies for Pharmaceutical and Biotechnological Applications* New York, Marcel Dekker, Ink., 615 p.

[6] Butt T. M., Jackson C., Magan N. (2001) *Fungi As Biocontrol Agents: Progress Problems and Potential* Wallingford, CABI Publishing, 393 p.

[7] Serrano A.B. (2015) Development of a rapid LC-MS/MS method for the determination of emerging Fusarium mycotoxins enniatins and beauvericin in human biological fluids. *Toxins*, No 7, pp. 3554-3571.

[8] Uhlig S., Ivanova L. (2004) Determination of beauvericin and four other enniatins in grain by liquid chromatography – mass spectrometry. *Journal of chromatography A*, No 1050, pp. 173-178. doi: 10.1016/j.chroma.2004.08.031.

[9] *PubMed Single Citation Matcher*. Available at: https://www.ncbi.nlm.nih.gov/pubmed/citmatch

[10] Bolechová M., Benesova K., Belakova S., Caslavsky J., Pospichalova M., Mikulikova R. (2015) Determination of seventeen mycotoxins in barley and wheat in the Czech Republic. *Food Control*, No 47, pp. 108-113. doi: 10.1016/j.foodcont.2014.06.045.

[11] Berthiller F. (2007) Chromatographic methods for the simultaneous determination of mycotoxins and their conjugates in cereals. *International Journal of Food Microbiology*, No 119, pp. 33-37.

[12] Jestoy M. (2008) Emerging fusarium-mycotoxins fusaproliferin, beauvericin, enniatins, and moniliformin: a review. *Critical review in food science and nutrition*, No 48, pp. 21-49. doi: 10.1080/10408390601062021.

[13] Habler K, Rychlik M. (2016) Multi-mycotoxin stable isotope dilution LC-MS/MS method for Fusarium toxins in cereals. *Analytical and Bioanalytical Chemistry*, No 408, pp. 307-317. doi: 10.1007/s00216-015-9110-7.

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Метод рідинної хроматографії – подвійної мас-спектрометрії для визначення еніатинів А, А1, Б, Б1 та бюварицину у озимій пшениці

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Мета: удосконалити та перевірити метод рідинної хроматографії – подвійної мас-спектрометрії для визначення мікотоксинів, що продукують гриби роду *Fusarium,* а саме еніатинів А, А1, Б, Б1 та бюварицину. **Методи:** у статті розглядається аналітичний метод, оснований на об'єднанні високоефективної рідинної хроматографії з подвійною мас-спектрометрією для швидкого розділення, ідентифікації та кількісного визначення мікотоксинів у зразку. **Результати:** доведено, що запропонований метод є точним, вибірковим та швидким для ідентифікації та визначення низькомолекулярних сполук, накопичених у зерні у малій кількості. **Обговорення:** підтверджено, що метод рідинної хроматографії – подвійної мас-спектрометрії є ефективним для визначення вмісту мікотоксинів у малих концентраціях. Діапазони валідації методу для аналітів порівняні із науковими роботами на подібну тему минулих років.

Ключові слова: бюварицин; гриби роду *Fusarium*; еніатини; мікотоксини; рідинна хроматографія – подвійна мас-спектрометрія.

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Метод жидкосной хроматографии – тандем масс-спектрометрии для определения энниатинов А, А1, Б, Б1 и бюварицина в озимой пшенице

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Цель: усовершенствование и проверка метода жидкосной хроматографии – тандем масс-спектрометрии для определения микотоксинов, продуцируемых грибами рода *Fusarium,* а именно энниетинов А, А1, Б, Б1 и бюварицина. **Методы:** в статье рассматривается аналитический метод, основанный на объединении высоко-эффективной жидкосной хроматографии с тандем масс-спектрометрией для быстрого разделения, идентификации и количественного определения микотоксинов в образцею **Результаты:** доказано, что предложенный метод есть точным, избирательным и быстрым для идентификации и определения низко-молекулярных соединений, накопленых в зерне у малых количествах. **Обсуждение:** подтвержено, что метод жидкосной хроматографии – тандем масс-спектрометрии является эффективным для определения содержания микотоксинов у малых концентрациях. Диапазон валидации методу для аналитов сравнен с научными работами на похожою тему прошлых годов.

Ключевые слова: бюварицин; гриби рода *Fusarium*; жидкосная хроматография – тандем масс-спектрометрия; микотоксины; энниатины.

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