Chapter 12

MODERN METHODS FOR DETERMINING MYCOTOXINS

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INTRODUCTION

Mycotoxins are the products of vital activity (metabolites) of microscopic fungi (molds). They can infect fodder plants during their vegetation and storage. Mycotoxins are present in almost all types of agricultural products worldwide[1].

Mycotoxins are the most dangerous for animal and human health. They are able to contaminate food and feed at all stages of production, storage, transportation and sale. They are natural contaminants of cereal grains, legumes, sunflower seeds, as well as vegetables and fruits. Studies conducted by both domestic and foreign scientists indicate the possibility of a high frequency and degree of contamination of food and feed[2]. Currently, about 350 species of microscopic fungi have been identified, which produce about 400 mycotoxins, most of which cause alimentary toxicosis in animals and humans. Mycotoxins are produced by different strains of fungi and each strain can produce several mycotoxins. A significant number of mycotoxins have immunosuppressive, mutagenic, allergenic, teratogenic and carcinogenic properties, contribute to a decrease in the overall resistance of the body, the development of infectious and non-invasive diseases. The presence of

[1]Slobodchikova I., & Vuckovic D. Liquid chromatography – high resolution mass spectrometry method for monitoring of 17 mycotoxins in human plasma for exposure studies. Journal of Chromatography A. 2018, 1548, 51–63. doi:10.1016/j.chroma.2018.03.030.

[2]Madalena M., Sobral C., Faria M. A., Cunha S. C., & Ferreira I. Toxicological interactions between mycotoxins from ubiquitous fungi: Impact on hepatic and intestinal human epithelial cells. Chemosphere. 2018, 202, 538–548. doi:10.1016/j.chemosphere.2018.03.122

mycotoxins in feed leads to a deterioration in productivity, reproduction and immune status of animals, causes a number of diseases [3, 4, 5, 6].

One of the indicators of food and feed safety is the study of the content of mycotoxins. Common mycotoxins are aflatoxins B_1, B_2, G_1, G_2 and deoxynivalenone. These toxins can affect grain crops, feed intended for farm animals, and finished food products[7].

Many world scientists are conducting research on improving, simplifying the process, reducing sample preparation time, reducing costs and simultaneously determining the maximum amount of mycotoxins in one sample preparation[8, 9, 10, 11, 12].

There are various methods for determining mycotoxins, but preference is given to arbitration quantitative confirmatory methods. For example, the high-performance liquid chromatography (HPLC) method is used to determine the content of aflatoxin B1 and the sum of aflatoxins B_1, B_2, G_1 and G_2 in grain

[3]Huang S., Zheng N., Fan C. Y., Cheng M., Wang S., Jabar A., Cheng J. B. Effects of aflatoxin B1 combined with ochratoxin A and/or zearalenone on metabolism, immune function, and antioxidant status in lactating dairy goats. Asian-Australasian Journal of Animal Sciences. 2018, 31 (4), 505-513. doi:10.5713/ajas.17.0279

[4]Huang X. J., Wang S. M., Mao D., Miao S., Hu Q., & Ji S. Optimized QuEChERS Method Combined with UHPLC-MS/MS for the Simultaneous Determination of 15 Mycotoxins in Liquorice. Journal of Aoac International. 2018, 101 (3), 633–642. doi:10.5740/jaoacint.17-0365

[5]Sieger M., Kos G., Sulyok M., Godejohann M., Krska R., & Mizaikoff B. Portable Infrared Laser Spectroscopy for On-site Mycotoxin Analysis. Scientific Reports. 2017, 7. doi:10.1038/srep44028

[6]da Silva E. O., Bracarense A., & Oswald I. P. Mycotoxins and oxidative stress: where are we? World Mycotoxin Journal. 2018, 11(1), 113–133. doi:10.3920/wmj2017.2267

[7]Adekoya I., Obadina A., Phoku J., De Boevre M., De Saeger S., & Njobeh P. Fungal and mycotoxin contamination of fermented foods from selected south african markets. Food Control. 2018, 90, 295–303. doi:10.1016/j.foodcont.2018.02.040.

[8]Soares R. R. G., Azevedo A. M., Fernandes P., Chu V., Conde J. P., Aires-Barros M. R. A simple method for point-of-need extraction, concentration and rapid multi-mycotoxin immunodetection in feeds using aqueous two-phase systems. Journal of Chromatography A. 2017, 1511, 15–24. doi:10.1016/j.chroma.2017.07.004

[9]Zhu R. Y., Zhao Z. Y., Wang J. H., Bai B., Wu A. B., Yan L. P., Song S. Q. A simple sample pretreatment method for multi-mycotoxin determination in eggs by liquid chromatography tandem mass spectrometry. Journal of Chromatography A. 2015, 1417, 1–7. doi:10.1016/j.chroma.2015.09.028

[10]Sun W. S., Han Z., Aerts J., Nie D., Jin M. T., Shi W., Wu A. B. A reliable liquid chromatography-tandem mass spectrometry method for simultaneous determination of multiple mycotoxins in fresh fish and dried seafoods. Journal of Chromatography A. 2015, 1387, 42–48. doi:10.1016/j.chroma.2015.01.071

[11]Karami-Osboo R., & Mirabolfathi M. A. Novel Dispersive Nanomagnetic Particle Solid-Phase Extraction Method to Determine Aflatoxins in Nut and Cereal Samples. Food Analytical Methods. 2017, 10 (12), 4086–4093. doi:10.1007/s12161-017-0975-2

[12]Banati H., Darvas B., Feher-Toth S., Czeh A., & Szekacs A. Determination of Mycotoxin Production of Fusarium Species in Genetically Modified Maize Varieties by Quantitative Flow Immunocytometry. Toxins. 2017, 9 (2). doi:10.3390/toxins9020070

crops[13]. Deoxynivalenol is also determined by the method of high-performance liquid chromatography using immunoaffinity columns, but Ukraine does not have a valid regulatory document for this method. Currently, Ukraine regulates the determination of deoxynivalenol only by the enzyme-linked immunosorbent assay method[14]. There are also European standardized methods for determining mycotoxins in various matrices (food products) [15, 16].

Harmonization of Ukrainian legislation with global standards and standards adopted in the EU requires the coordination of national requirements for the control of feed safety for productive animals, which will make it possible to export agricultural products outside Ukraine. The requirements for methods for controlling the content of mycotoxins in feed materials and feed should be revised accordingly.

Nowadays, in connection with the harmonization of Ukrainian legislation with EU requirements and Ukraine's membership in the WTO, the implementation of a quality system in independent laboratories and laboratories at food and feed production facilities is becoming particularly relevant. In particular, this applies to chemical-analytical testing laboratories that are involved in the control of food safety and quality[17]. It should be noted that in the countries of the European Union the requirements for the content of foreign and toxic compounds in food are quite strict[18]. In this case, the effectiveness and reproducibility of each method used for the analysis of food and feed must be proven[19]. This can be achieved by assessing the suitability (validation) of the method according to generally accepted and proven approaches. These criteria are presented in Commission Decision

[13] ДСТУ EN 12955-2001 Продукти харчові. Визначення афлатоксину-В1 та суми афлатоксинів В1, В2, G1 та G2 у зернових культурах, фруктах із твердою шкіркою та похідних від них продуктах. Метод високоефективної рідинної хроматографії за допомогою постколонкової дериватизації та очищання на імунній колонці (EN 12955:1999, IDT)

[14] ДСТУ 8168:2015 Зернові культури, продукти їх переробляння, комбікорми. Метод визначення вмісту дезоксиніваленолу

[15] EN 15891:2010. Foodstuffs – Determination of deoxynivalenol in cereals, cereal products and cereal based foods for infants and young children – HPLC method with immunoaffinity column cleanup and UV detection

[16] EN 15791:2009. Animal feeding stuffs - Determination of Deoxynivalenol in animal feed - HPLC method with UV detection and immunoaffinity column clean-up

[17] Бережна Л.Х., Цвіліховський В.І., Кліментьєва Л.В. Основні валідаційні характеристики методу «Охрапреп» Кількісне визначення охратоксину А з використанням високоефективної рідинної хроматографії. Журнал хроматографічного товариства. 2008, VIII, 3,4, 34–38

[18] Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. Official Journal of the European Union. 20.12.2006. L 364/5

[19] Новожицька Ю.М., Іванова О.В., Доброжан Ю.В. Оцінка придатності підтверджуючих методів для визначення залишкових кількостей ветеринарних препаратів у продуктах тваринного походження. Науковий вісник ветеринарної медицини. 2015, 2, 14–18

2002/657/EC[20]. This regulatory legal act contains rules for chemical-analytical methods used in the examination of samples taken in accordance with the second paragraph of Article 15 of Directive 96/23/EC and defines common criteria for the interpretation of the results obtained in laboratories. Given the consistent, detailed and clear presentation of the validation procedure, the selection of its criteria and the processing of the data obtained, Commission Decision 2002/657/EC can be used as a basis for the validation of any analytical research method.

The aim of the work was to determine the suitability of methods for detecting residual amounts of aflatoxin B1 and deoxynivalenol in grain and feed by high-performance liquid chromatography according to validation criteria: linearity, detection limit, specificity, intra-laboratory reproducibility, accuracy (recovery).

1. Materials and methods of research

The research was conducted on the basis of the Ukrainian Laboratory of Quality and Safety of Agricultural and Industrial Products of the National University of Life and Environmental Sciences of Ukraine, which includes a research center for monitoring the quality and safety of agricultural resources and agricultural and industrial products. It is equipped with modern measuring equipment, automated computer systems, and numerous databases. The quality management system of Laboratory is built in accordance with the requirements of DSTU ISO/IEC 17025:2006 (ISO/IEC 17025:2005), which is confirmed by the Accreditation Certificate of the National Accreditation Agency of Ukraine.

When assessing the suitability of the method for determining the content of aflatoxin B_1 in grain crops by high-performance liquid chromatography (HPLC), a Shimadzu LC-20A liquid chromatograph with a fluorescent detector was used. For the analysis, analytical reversed-phase columns C18 were used: Supelco Ascentis™, length 150 mm, internal diameter 4.6 mm, sorbent with a particle size of 5.0 μm and a reversed-phase chromatographic precolumn Supelguard™ Ascentis™, length 20.0 mm, internal diameter 4.0 mm, sorbent with a particle size of 5.0 μm, 5 um kit.

To assess the suitability of the method for determining the content of deoxynivalenol (DON) in grain crops by HPLC with purification on immunoaffinity columns, an Ultimate 3000 liquid chromatograph with a diode-array detector was used. For the analysis, a Supelco Discovery C18

[20]Commission Decision of 14 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results (notified under document number C (2002) 3044), text with EEA relevance 2002/657/EC. // Official Journal of the European Union. 17.08.2002. L 221. P. 8.

chromatographic column, length 250.0 mm, internal diameter 4.6 mm, particle size 5.0 μm, was used.

The mobile phase for the determination of aflatoxin B_1 was acetonitrile: water: methanol (2:6:2), containing potassium bromide at a concentration of 0.12 g/l and 200 μl/l nitric acid; for the determination of deoxynivalenol, the mobile phase was acetonitrile:water:methanol (3:94:3).

Chromatographic analysis conditions for the determination of aflatoxin B1:

1) flow rate: 1 cm^3/min;
2) injection volume: 20 μl;
3) column thermostat temperature: 40°C;
4) wavelengths: λex = 362 nm; λem = 440 nm.

Chromatographic analysis conditions for the determination of deoxynivalenol:

1) flow rate: 1 cm^3/min;
2) injection volume: 20 μl;
3) column thermostat temperature: 30°C;
4) autosampler temperature: + 13°C
5) wavelength: 218 nm.

The control was cereal grain samples (wheat, barley), which were previously checked for the absence of target analytes. The introduction of mycotoxins into the control samples to the required concentration level was carried out using standard solutions. A standard certified sample of aflatoxin B_1 with a concentration of 25 μg/ml manufactured by Trilogy and a standard sample of deoxynivalenol with a concentration of 1000 μg/ml manufactured by Sigma-aldrich were used. The standard samples and test samples were stored under appropriate microclimate conditions in the sample preparation rooms: temperature – 20±5°C, humidity – up to 80%. In parallel, samples were prepared with the addition of the corresponding mycotoxins and the purity of the reagents was controlled.

The work used immunoaffinity columns AFLAPREP® and DONPREP®, designed for the isolation of aflatoxins B_1, B_2, G_1 and G_2 and deoxynivalenol from cereals, manufactured by R-BIOPHARM RHONE LTD. The study was conducted in accordance with the methodological recommendations for the use of these columns[21],[22].

Methodology for the determination of aflatoxin B_1. Since DSTU EN 12955-2001 does not clearly specify the type and manufacturers of immunoaffinity columns that must be used during the analysis, we used

[21]AFLAPREP®. Immunoaffinity columns for use in conjunction with HPLC or LC-MS/MS. For in vitro use only. R-BIOPHARM RHÔNE LTD, 2016, 23 p.

[22]DONPREP® Immunoaffinity columns for use in conjunction with HPLC or LC-MS/MS. For in vitro use only. R-BIOPHARM RHÔNE LTD, 2016, 23 p.

AFLAPREP® columns to develop the method, which make the sample purification procedure more convenient. At the same time, the sensitivity of the method increases due to enrichment of the analysis and its purification from matrix components, and the analysis speed increases. Therefore, the working method proposed by us follows from the AFLAPREP® column used by us. For this, 50 g of homogenized sample was weighed and 5 g of sodium chloride and 100 cm3 of 80% methanol were added.

To confirm the presence of aflatoxin B_1 in the sample by HPLC, it is necessary to perform derivatization, which increases the natural fluorescence of the toxin under UV light and makes their detection easier. Traditionally, complex chemical derivatization is used for this purpose. However, it has significant limitations that can be overcome using the KOBRA well.

During electrochemical derivatization of aflatoxin B_1, a bromide binding reaction occurs in the KOBRA well (reaction time 4 sec at room temperature). This does not require additional equipment and daily preparation of reagents, unlike the use of iodine as a derivative.

Therefore, the assessment of the suitability and validation of the working method was carried out taking into account the above-mentioned immunoaffinity columns and the KOBRA well used by us. According to the methodological recommendations for the use of AFLAPREP® immunoaffinity columns and DSTU EN 12955-2001, the procedure for assessing the feasibility of the method includes the following main stages: extraction with methanol, filtration, retention of aflatoxins, washing, elution, determination of aflatoxins using high-performance chromatography. Further validation showed that the proposed working method is optimal for the Shimadzu liquid chromatograph (Japan) in the conditions of this laboratory.

The determination of deoxynivalenol according to the method developed by us includes the following main stages: extraction with deionized water, filtration (centrifugation), retention of deoxynivalenol in the DONPREP® immunoaffinity column, washing of the DONPREP® immunoaffinity column, elution of deoxynivalenol from the DONPREP® immunoaffinity column, evaporation of the eluate, dilution with a mobile phase, determination of deoxynivalenol using high-performance chromatography. In this case, 25 g of finely ground barley sample was taken, 5 g of sodium chloride was added, 200 cm^3 of distilled water was added and extracted for 30 min on a shaker. The extract was filtered and passed through the DONPREP® immunoaffinity column. The method was tested on a Dionex ICS 3000 liquid chromatograph (USA). The assessment of the suitability of the methods was carried out in accordance with Commission Decision 2002/657/EC, which implements Council Directive 96/23/EC on the performance of analytical methods and the interpretation of results.

2. Assessment of the conformity of the methods for aflatoxin B_1 and deoxynivalenol determination in grain and feeds by method of high-performance liquid chromatography

The assessment of validation criteria (linearity, detection limit, quantification limit, specificity, intralaboratory reproducibility, accuracy) and their parameters began with determining the type of method chosen. In this case, the HPLC method is quantitative. It is an analytical method that determines the amount or mass fraction of a substance in such a way that it can be expressed as a numerical value of the corresponding units. One of the most important criteria of a quantitative analytical method is accuracy – the degree of closeness between the average value obtained from a series of research results and the accepted value. This criterion reflects how closely the obtained result corresponds to the actual value and for its determination, researchers are required to use certified reference material. In its absence, it is allowed to use the method of adding standards to a clean matrix with subsequent analysis determination and the corresponding calculation of the percentage of recovery (P). According to the recommendations of the above-mentioned decision for substances with established maximum permissible levels (MRLs), the sample should be fortified with the analyte in three concentrations, which are 0.5; 1 and 1.5 of the MRL, ten samples for each. The MRL of aflatoxin B_1 according to Commission Regulation EC No. 1881/2006 for cereals and cereal products is 2 μg/kg.

The matrix (barley flour) was analyzed for the content/absence of aflatoxin B_1 by operator I. According to the data obtained, aflatoxin B1 is absent in the matrix.

The main validation criteria for the quantitative confirmatory method for *the determination of aflatoxin B_1* in cereals according to Commission Decision 2002/657/EC are:

– linearity (calibration curve);
– limit of detection (LOD);
– limit of quantification (LOQ);
– specificity (selectivity);
– intralaboratory reproducibility;
– accuracy (recovery).

Definition of linearity. Linearity is the ability of a method (within the range of application) to give values directly proportional to the concentration (amount) of the analyte in the sample.

Using the available standard solutions of aflatoxins B_1, ten concentrations were prepared, including zero – 0 ng/cm^3; 0.05 ng/cm^3; 0.1 ng/cm^3; 0.5 ng/cm^3; 1.0 ng/cm^3; 2.0 ng/cm^3; 5.0 ng/cm^3; 10.0 ng/cm^3; 20.0 ng/cm^3; 40.0 ng/cm^3 (at least in three parallels).

According to the obtained data, which were processed by the Spline program (Table 1), the response of the instrument to the studied analyte concentrations is linear and the method can be used for the quantitative determination of aflatoxin B_1 in experimental grain samples.

Table 1

Regression analysis for the method for determining the content of aflatoxin B_1

Regression	**a + bx**
a =	0.0245 (95% confidence interval a ± 0,0296)
b =	1.0017 (95% confidence interval b ± 0,0299)
Residual variance Sy:	0.0355
Standard square deviation (SSD) of the method (Sxo=Sy/b):	0.0354
Coefficient of variation Vxo = Sxo/Xcp,(%):	10.779
Detection limit:	0.0335 (Xc=0,0167) concentration, ng/cm^3
Convergence criterion (d) for N=2,3,4 (%):	0.9997
Data linearity:	Satisfactorily {PG=0.1813; F(1;7;0.99)=5.592}
Homogeneity of dispersions:	Satisfactorily {PG=2.945; X2(8;0.95)=15.51}

The detection limit for an analytical instrument is the lowest concentration of the analyte that can be identified on a given instrument by a given method with acceptable statistical probability.

According to the obtained data, which were processed by the Spline program (Table 1), the detection limit for a given analytical standard on the equipment used is 0.03 ng/ml of aflatoxin B_1 in solution. Therefore, the detection limit of aflatoxin B_1 is acceptable.

The limit of quantification of a method is the lowest concentration of the analyte in the sample (μg/kg) that can be measured by this method with acceptable statistical reliability and a defined uncertainty.

According to Commission Regulation EC 1881/2006, the MRL for aflatoxin B1 for all cereals and all cereal products is 2.0 μg/kg. According to this directive, the lowest concentration of aflatoxin B_1 that is 0.5 of the MRL is 1.0 μg/kg (Table 2).

Table 2

Results of intra-laboratory reproducibility for the quantitative confirmatory method for the determination of aflatoxin B1, n=20

Aflatoxin B_1 concentration (K)						
Operator number	**1 μg/kg added**		**2 μg/kg added**		**3 μg/kg added**	
	K, ng/ml	K_1, μg/kg	K, ng/ml	K_1, μg/kg	K, ng/ml	K_1, μg/kg
$K_{average}$	0.45	0.89	0.84	1.67	1.26	2.53
Standard deviation	0.01	0.02	0.02	0.05	0.02	0.04
CV,%	2.34	2.34	2.68	2.68	1.38	1.38
$CV_{mean} = 2.13 \pm 0.67$						

According to the data obtained by us, the limit of quantification for this method on the equipment used is 0.89 μg/kg of aflatoxin B_1, which meets the requirements of the directive.

Specificity and selectivity are the properties of a method to accurately determine the analyte of interest in the presence of other components in the sample under standard test conditions.

To determine these indicators, 10 independent samples of a blank matrix (barley flour) were analyzed. Aflatoxin B_1 was not detected in them, therefore, the criteria of specificity and selectivity are validated (confirmed).

Reproducibility is precision under reproducibility conditions, i.e. under conditions where test results are obtained by the same method on identical test samples in different laboratories, by different operators, on different equipment.

Intra-laboratory reproducibility reflects the ability of the method to provide repeatable results with a small statistical deviation under the influence of minor changes (replacement of reagents, room temperature, conducting the study on a different day, on a different device, etc.).

Intra-laboratory reproducibility reflects the variation of results observed when one or more factors are changed in the same laboratory. One of the factors may also be the same sample over a long period of time (repeated measurements), but only if it is reliably known that the parameter being measured does not change its values over time.

To determine this criterion, the following manipulations were performed:

– the blank matrix (barley flour) was enriched with aflatoxin B_1 in concentrations equivalent to 0.5; 1 and 1.5 MRL, i.e.: 1.0; 2.0 and 3.0 μg/kg. Each of the above concentrations was analyzed in 10 parallels.

This experiment was reproduced similarly under other conditions (change in ambient temperature, reagent batches) and with a different operator.

After that, the concentration of aflatoxin B_1 was calculated in each analyzed sample and the average concentration, standard deviation and

coefficient of variation (CV,%) in the enriched samples were calculated (formula 1):

$$CV = CB/Kc \cdot 100,\%, \quad (1)$$

where SV is the standard deviation; Kc is the average value of the obtained analyte concentration.

The calculations were made according to formula 2:

$$K_1 = K \cdot 2, \mu g/kg, \quad (2)$$

where K_1 is the concentration of aflatoxin B_1, μg/kg; K is the concentration of aflatoxin B1 obtained as a result of chromatographic analysis of the sample, ng/cm^3; K·2, since K is the concentration in 1 cm^3, we obtain the concentration in ng/cm^3, and 2 is the conversion coefficient, according to the method, which is identical to μg/kg.

For a more complete assessment of the obtained data, the coefficient of variation was calculated, which characterizes the relative degree of deviation of the measured values from the arithmetic mean.

The coefficient of variation (CV) was calculated for the purpose of comparative analysis of results obtained by two operators. CV characterizes intra-laboratory reproducibility and is: for a concentration of 1 μg/kg – 2.34%; for a concentration of 2 μg/kg – 2.68%; for a concentration of 3 μg/kg – 1.38%. CV_{mean} = 2.13±0.67, which according to Commission Decision 2002/657/EC should not be > 20%, and therefore is appropriate.

Accuracy – reflects the closeness of a large number of measurement results to the true (actual) or accepted reference value.

The accuracy is determined by the following methods: using certified standard samples (in the case of repeated measurements of certified reference material, the deviation ranges given in Table 3 for the experimentally determined average mass fraction value from the certified value should be taken into account.

Table 3

Minimum recovery of quantitative methods

Mass fraction	Range
≤ 1 μg/kg	from minus 50% to +20%
>1 μg/kg – 10 μg/kg	from minus 30% to +10%
≥ 10 μg/kg	from minus 20% to +10%

In the case where reference materials are not available, accuracy can be assessed by determining the recovery from the addition of known amounts of the substance under investigation to a blank matrix. The quantitative indicator of accuracy in this case will be the percentage recovery.

We evaluate the recovery, which determines the accuracy of the measurement procedure.

Recovery is the percentage of the true concentration of the analyte that is recovered during the analytical method. It is determined during the validation process if a certified reference material is not available.

Recovery was determined by adding the active ingredient to a blank sample. The exact amount of the active ingredient (aflatoxin B_1) was added to the blank sample at concentrations of 0.5; 1.0; 1.5 MRL, respectively.

The recovery percentage is calculated as follows (formula 3):

$$RP = \frac{\text{returned quantity}}{\text{added quantity}} \cdot 100\,\%, \tag{3}$$

Confidence interval for the recovery percentage (formula 4):

$$RP - (95\%, n-10); (\frac{x - 2{,}23 \cdot s/\sqrt{10}}{\text{added quantity}} \cdot 100); (\frac{x + 2{,}23 \cdot s/\sqrt{10}}{\text{added quantity}} \cdot 100), \tag{4}$$

where x is the average of 10 determinations; s is the standard deviation (σ_{n-1}).

The assessment of the *recovery* criterion for the suitability of the method for determining the content of aflatoxin B_1 was determined according to the following scheme:

– 10 blank samples were analyzed to ensure the purity of the matrix (the data obtained during the determination of the previous parameters can be used) and 10 aliquots were fortified at 0.5; 1.0 and 1.5 MRL, i.e. 1.0; 2.0 and 3.0 μg/kg. The concentration present in each sample was determined;

– the recovery for each sample was determined using formula (3) above;

– calculated the mean value of the return and the coefficient of variation (CV = SV/Kc·100) from ten results for each level.

20 samples of barley flour were analyzed for each concentration: 1 μg/kg (0.5 MRL); 2 μg/kg (1 MRL) and 3 μg/kg (1.5 MRL). The calculations were made according to the following formula:

$$K = (C \cdot 2)/1000) \cdot 100\,0, \tag{5}$$

where K is the concentration of aflatoxin B_1, μg/kg of flour; C is the concentration of aflatoxin B_1 in the sample, ng/cm^3 (according to calibration solutions); 2 is the conversion factor according to the method; 1000 is the conversion factor from nanograms to micrograms; 1000 is the conversion factor from mass to 1 kg.

According to the data obtained for the added concentration of aflatoxin B_1 (1 μg/kg), the actual obtained concentration is 0.89±0.02 μg/kg (Table 4).

In this case, the recovery percentage is 89.4±2.34%, which corresponds to the minimum permissible values (for concentrations > 1 μg/kg – 10 μg/kg, the recovery should be 70–110%).

The coefficient of variation for this added concentration is 2.34%, which according to Commission Decision 2002/657/EC is appropriate, since it is not > 20%.

Table 4

Mean values of the recovery results for aflatoxin B1 in three concentrations, n=20

Aflatoxin B_1 concentration (K)						
Criteria	**1 μg/kg added**		**2 μg/kg added**		**3 μg/kg added**	
	C, ng/ml	**K, μg/kg**	**C, ng/ml**	**K, мкг/кг**	**C, ng/ml**	**K, μg/kg**
$K_{average}$	0.45	0.89	0.84	1.67	1.26	2.53
Standard deviation	0.01	0.02	0.02	0.05	0.02	0.04
CV,%	2.34	2.34	2.68	2.68	1.38	1.38
Recovery,%	-	89.4	-	83.7	-	84.3

According to the data obtained for the added concentration of aflatoxin B_1 (2 μg/kg), the actual obtained concentration is 1.67±0.05 μg/kg; with a recovery rate of 83.7±2.68%, which is within the minimum permissible limits.

The coefficient of variation for the added concentration is 2.68%, which is appropriate.

According to the data obtained, for an added concentration of 3 μg/kg of aflatoxin B_1, the actual concentration obtained is 2.53±0.04 μg/kg; with a recovery rate of 84.3±1.38%, which is within the minimum permissible limits.

The coefficient of variation for an added concentration of 3 μg/kg is 1.38%, which is appropriate.

Therefore, according to the obtained data, for all analyzed concentrations of aflatoxin B_1, the recovery percentage is 85.8±3.65%, which corresponds to the minimum permissible values (for concentrations ≥ 1–10 μg/kg, the recovery percentage should be 70–110%), while the coefficient of variation (CV,%) is 2.13 ± 0.67 and is appropriate, since according to Commission Decision 2002/657/EC it should not be > 20% (Table 5).

The next stage of our work was to assess the suitability of the measurement method for deoxynivalenol.

Due to the fact that Ukraine does not have a national standard for the determination of deoxynivalenol by HPLC, a working method for the determination of deoxynivalenol content in grain crops by high-performance liquid chromatography with purification on immunoaffinity columns was developed, tested and validated. The use of specific immunoaffinity columns simplifies and accelerates the complex procedure of sample preparation in chromatographic analysis, makes it possible to accurately determine the amount of this mycotoxin in the sample after the extraction procedure. Using immunoaffinity columns, the analyte is enriched and purified from matrix components. Advantages of immunoaffinity columns: improved sample preparation quality, increased sensitivity of the HPLC method, speed of analysis, convenience of the sample purification procedure.

Table 5

Metrological characteristics of the assessment of the suitability of the measurement method for aflatoxin B_1

Validation criterion according to Directive EC 657/2002	Criteria requirements according to Directive EC 657/2002	Result obtained	Conformity
Reproducibility (intra-laboratory)	CV ≤ 20%	Concentration 1 μg/kg: CV = 2.34%. Concentration 2 μg/kg: CV = 2.68%. Concentration 3 μg/kg: CV = 1.38%. CV_{mean} = 2.13 ± 0.67%	consist
Calibration curves	Linearity at the tested concentrations	The response of the instrument at the tested analyte concentrations (0–40 ng/mL) is linear	consist
LOD	Individual for individual equipment	0.03 ng/ml	consist
LOQ	Individual for a particular method	0.89 μg/kg	consist
Recovery	Recovery: 70% – 110% CV ≤ 20%	Concentration 1 μg/kg: Recovery 89.4 ± 2.34%, CV = 2.34%. Concentration 2 μg/kg: Recovery 83.7 ± 2.68% CV = 2.68%. Concentration 3 μg/kg: Recovery 84.3 ± 1.38%; CV = 1.38%; Mean recovery: 85.8 ± 3.65%; CV_{mean} = 3.65%.	consist

In order to assess the suitability of the measurement method for deoxynivalenol, the same criteria were used as for aflatoxin B_1.

The matrix (barley flour) was analyzed for the presence/absence of deoxynivalenol by operator I. According to the obtained data, deoxynivalenol was detected in the matrix in an amount of 0.0438 mg/kg.

For deoxynivalenol, according to Commission Regulation (EC) 1881/2006, the MRL or permitted limit for whole barley grain is 1.25 mg/kg, and 0.75 mg/kg for its processed products. According to the Mandatory Minimum List of Research on Raw Materials, Products of Animal and Plant Origin, Compound Feed Raw Materials, Compound Feeds, Vitamin Preparations, etc., the MRL for deoxynivalenol in animal feed is 1.0 mg/kg.

According to the current DSTU 3769-98, the MRL for barley used for industrial purposes and for export is 1.0 mg/kg, for barley used for feed purposes – 0.5 mg/kg [23], [24].

Linearity (calibration curve).

From the available standard solutions of deoxynivalenol, 7 concentrations were prepared, taking into account zero – 0 μg/cm^3; 0.0625 μg/cm^3; 0.125 μg/cm^3; 0.250 μg/cm^3; 0.5 μg/cm^3; 1.0 μg/cm^3; 2.0 μg/cm^3, which were studied in three parallels.

According to the obtained data, which were processed by the Spline program (Table 6), the response of the instrument to the studied analyte concentrations is linear, therefore the method can be used for the quantitative determination of deoxynivalenol in samples.

The detection limit for this analytical standard on the equipment used is 0.009 mg/cm^3 of deoxynivalenol in solution. Therefore, the detection limit of deoxynivalenol is acceptable.

Limit of quantification.

According to the current DSTU 3769-98 the MRL for barley is 0.5 mg/kg. During the experiment, the lowest concentration of deoxynivalenol in the barley sample was established (0.24±0.02 mg/kg), which can be quantitatively calculated using the Ultimate 3000 liquid chromatograph with a diode array detector (Table 6).

According to the data obtained, the limit of quantification (LOQ) for this method on the equipment used is 0.24 mg/kg of deoxynivalenol.

Specificity and selectivity

To determine these indicators, 10 independent samples of the blank matrix (barley flour) were analyzed: the average concentration of deoxynivalenol was 0.0438 mg/kg, the standard deviation (SD) was 0.0039.

The average value of the 10 analyzed independent samples of the blank matrix is ≈ 43.8 μg/kg. In the future, to determine criteria such as intra-laboratory reproducibility and percentage recovery during blank soldering, it is necessary to take into account the specified value of the blank concentration.

[23]ДСТУ 3769-98 Ячмінь. Технічні умови. З Поправкою (ІПС № 6-99)

[24]Обов'язковий мінімальний перелік досліджень сировини, продукції рослинного та тваринного походження, комбікормової сировини, комбікормів, вітамінних препаратів та ін. Наказ Державного департаменту ветеринарної медицини України від 03.11.1998 року, №16.

Table 6

Regression analysis for the method for determining the content of deoxynivalenol

Regression	a + bx
a =	0.0005 (95% confidence interval a ± 0.0023)
b =	0.5121 (95% confidence interval b ± 0.0057)
Residual variance Sy:	0.002
Standard square deviation (SSD) of the method (Sxo=Sy/b):	0.004
Coefficient of variation Vxo = Sxo/Xcp, (%):	1.897
Detection limit:	0.009 (Xc=0.004) concentration, μg/cm^3
Convergence criterion (d) for N=2,3,4 (%):	0.9999
Data linearity:	Satisfactorily {PG=0.2696; F(1;5;0.99)=6.608}
Homogeneity of dispersions:	Satisfactorily {PG=-29.89; X2(6;0.95)=12.59}

Intra-laboratory reproducibility was performed according to the following scheme:

– the blank matrix (barley flour) was spiked with deoxynivalenol at concentrations equivalent to 0.5; 1.0 and 1.5 MRL, i.e.: 250, 500 and 750 μg/kg. Each concentration was analyzed in 10 parallels;

– this experiment was repeated under different conditions (change in ambient temperature, reagent batches) and with a different operator.

After that, the concentration of each analyzed sample was calculated and the average concentration, standard deviation and coefficient of variation (CV%) in the spiked samples were calculated according to formula 6.

$$K = (C \cdot 1000)/1 \quad (6)$$

where K is the DON concentration, mg/kg of flour; C is the DON concentration in the sample, μg/cm^3 (according to calibration); 1000 is the conversion to μg/kg; 1 is the conversion factor under the condition of passing 8 cm^3 of extract through the column (equivalent to 1 g of sample).

The coefficient of variation was calculated for the purpose of comparative analysis of the results obtained by two operators. The coefficient of variation (CV) characterizes intra-laboratory reproducibility and is: for a concentration of 250 μg/kg – 8.7%; for a concentration of 500 μg/kg – 11.3%; for a concentration of 750 μg/kg – 8.2%. $CV_{mean} = 9.4 \pm 1.65$ is appropriate, since according to Commission Decision 2002/657/EC it should not be > 20%.

Recovery. The recovery was determined by adding the active ingredient to the blank sample. The exact amount of the active ingredient (analyte) was

added to the blank sample at concentrations of 0.5; 1.0; 1.5 MRL, respectively, measured once.

The recovery percentage was calculated according to formula 3.

The confidence interval for the recovery percentage was according to formula 4.

During the assessment of the suitability of the method for the determination of deoxynivalenol, the recovery percentage was determined according to the following scheme:

– 10 blank samples were analyzed to ensure the purity of the matrix and 10 aliquots were fortified at the level of 0.5; 1.0 and 1.5 MRL, i.e. at the level of 250; 500 and 750 μg/kg. The concentration present in each sample was determined;

– the recovery was determined for each sample using formula 3 above;

– the average recovery and coefficient of variation (CV = SV/Kc·100) were calculated from ten results for each level.

20 samples of barley flour were analyzed for each concentration: 250 μg/kg (0.5 MRL); 500 μg/kg (1.0 MRL) and 750 μg/kg (1.5 MRL). The calculations were made according to formula 6/

According to the data obtained (Table 7), for the added concentration of deoxynivalenol (250 μg/kg), the actual concentration obtained is 236.7±8.7 μg/kg; the recovery percentage is 94.7±8.2%, which corresponds to the minimum permissible values according to Commission Decision 2002/657/EC (for concentrations ≥ 10 μg/kg, the recovery percentage should be 80–110%).

The coefficient of variation for this added concentration is 8.7%, which should not be > 20%, and therefore is appropriate.

Table 7

Mean values of the recovery results for deoxynivalenol at three concentrations, n=20

Deoxynivalenol concentration			
Criteria	**250 μg/kg**	**500 μg/kg**	**750 μg/kg**
C_{mean}, μg/kg	236.7	444.5	630.8
C_{mean}, μg/kg	2.37	4.45	6.3
CB	20.6	50.2	52.0
CV,%	8.7	11.3	8.2
Recovery,%	94.7	88.9	84.1

According to the data obtained for the added concentration of deoxynivalenol (500 μg/kg), the actual obtained concentration is 444.5±11.3 μg/kg; while the recovery percentage is 88.9±10.0%, which corresponds to the minimum permissible values.

The coefficient of variation for this added concentration is 11.3%, which is appropriate.

For the added concentration of deoxynivalenol (750 μg/kg), the actual obtained concentration is 630.8±8.2 μg/kg; while the recovery percentage is 84.1±10.0%, which corresponds to the minimum permissible values. The coefficient of variation for this added concentration is 8.2%, which is appropriate.

Therefore, according to the obtained data, for all analyzed concentrations of deoxynivalenol, the recovery percentage is 89.2±5.3%, which corresponds to the minimum permissible values according to Commission Decision 2002/657/EC (for concentrations ≥ 10 μg/kg, the recovery percentage should be 80–110%), while the coefficient of variation (CV,%) is 9.4±1.65, which is appropriate.

CONCLUSIONS

Based on the conducted experimental studies, it was established that the methods for determining the content of aflatoxin B1 by high-performance liquid chromatography and the determination of the content of deoxynivalenol by high-performance liquid chromatography with purification on immunoaffinity columns are suitable for the study of grain, grain products and feed from it and can be used by laboratories to conduct similar studies. The adapted methods for determining mycotoxins are highly sensitive and their parameters meet European requirements.

SUMMARY

The suitability of methods for determination of residual amounts of aflatoxin B_1 and deoxynivalenol in grain, products and feeds from grain by the method of high-performance liquid chromatography with validation criteria was evaluated: linearity, detection limit, specificity, intralaboratory reproducibility, correctness (return).

It was concluded that the methods for determining in cereal cultures of aflatoxin B_1 and deoxynivalenol content with purification on immunoaffinity columns by the method of high-performance liquid chromatography are suitable for the study of grain, products and feeds from grain and can be used by laboratories for conducting similar studies.

Adapted methods of mycotoxins determination are highly sensitive and meet European requirements according to their parameters.

The recovery percentage is 85.8 ± 3.65% for all the concentrations of aflatoxin B_1 analyzed. It corresponds to the minimum allowable value according to Commission Decision 2002/657/EC, with the coefficient of variation (CV,%) being 2.13 ± 0.67 and is in accordance with Commission Decision 2002/657/EC. The recovery rate is 89.2 ± 5.3% for all analyzed concentrations of deoxynivalenol, which corresponds to the minimum

allowable value according to Commission Decision 2002/657/EC, the coefficient of variation (CV,%) is 9.4 ± 1.65 and is suitable.

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