

**МІКРОБІОЛОГІЯ, ЕПІЗООТОЛОГІЯ,  
ІНФЕКЦІЙНІ ХВОРОБИ ТА ІМУНОЛОГІЯ**

**UDC 579.8:543.544.74**

**BILAN A.**, Candidat of veterinary sciences  
*Bila Tserkva State Agrarian University*

**THE SEPARATION AND DETECTION OF SEVERAL  
MYCOTOXINS BY THIN-LAYER CHROMATOGRAPHY**

В статті описано розробку нової системи для одночасного розділення і виявлення одинадцяти різних мікотоксинів. Водорозчинні та ліпідні домішки видаляються з екстракту за допомогою рідинного розділу. Для сумішей, що містять кислі мікотоксини, наприклад циклопіазонову кислоту, секалонову D кислоту, охратоксини А і В, очищення досягається шляхом видалення нейтрального матеріалу за стандартною методикою. Для аналізу афлатоксинів, охратоксину, зearаленону, цитриніну, патуліну, трихотеценів, циклопіазонові кислоти, рубратоксину, стеригматоцистину, пеніцилової кислоти, бутеноліду та цитреовіридину використовували ТШХ. Для дослідів застосовували силікагель G (Merck), суспендований в 0,4 N водному розчині щавлевої кислоти в співвідношенні (1:2). Поєднанням розчинником був хлороформ-метил-ізобутилкетон (4:1). Плями мікотоксинів виявляли під УФ-світлом з довжиною хвилі (366 Нм) із розпиленням кольорових реагентів.

**Ключові слова:** мікотоксини, екстракти, очищення, тонкошарова хроматографія, виявлення.

**Statement of the problem.** The analysis of mycotoxins involves a sequence of discrete operations which includes sampling, sample preparation, extraction, clean-up, quantification and confirmation procedures. Since mycotoxins occur in a wide variety of commodities and products, the analyst is faced with the problem of removing a large number of disparate, interfering compounds from the sample extracts [1]. The study of mycotoxicoses especially aflatoxicosis emphasized the existence of fungal metabolites harmful to higher organisms [2, 3].

**Analysis of recent research and publications.** This evidence and the ability of various ubiquitous fungi *Aspergillus flavus* [3] and *Penicillium islandicum* [4] to elaborate potent carcinogens prompted theories on a possible relationship between the consumption of mycotoxins and diseases of unknown etiology, e.g. the high incidence of hepatocarcinogenicity in Africa [5]. It is therefore essential that rapid and sensitive analytical methods be developed for the detection of these hazardous compounds in agricultural commodities and consumer products. These methods can be used to study the factors which would influence the growth of the toxigenic fungi in nature and their production of mycotoxins. It is of importance to note that the presence only of toxigenic fungi on a specific product does not necessarily indicate the presence of any mycotoxin.

The main purpose of research. Several excellent methods have been reported for the screening and quantitative estimation of the aflatoxins [6] introduced a method for the screening for zearalenone, aflatoxin and ochratoxin [7].

**Materials and methods of the study.** A normal TLC plate consists of a thin, uniform layer of particulate adsorbent, the stationary phase, applied to a flat plate. Silica, alumina and cellulose are frequently used as stationary phases. The chromatography is performed by the dropwise application of microlitre quantities, of a solution of the cleaned-up analyte mixture, to one end of the TLC plate. The mixture is drawn through the stationary phase, by capillary action, within the developing solvent (the mobile phase), which is usually contained within a sealed glass tank. During this chromatographic process, the components of the analyte mixture are partitioned between the stationary and mobile phases. The components of greater polarity will have the greater affinity for the stationary phase and will travel more slowly through the adsorbent, thus effecting the separation of the analyte mixture.

Both one dimensional and 2D TLC have been applied, with great success to the analysis of mycotoxins. TLC, for example, has been applied to the analysis of the aflatoxins, the ochratoxins, zearalenone, citrinin, patulin, the trichothecenes, cyclopiazonic acid, the rubratoxins, sterigmatocystin, penicillic acid, butenolide and citreoviridin.

Silica Gel G (Merck) was slurried with 0,4 N aqueous oxalic acid in a (1:2) ratio. The separation was achieved on 20×20 cm plates using an 0.25 mm layer of the above-mentioned slurry. The plates were air dried, activated at 100° for 40 min and kept at room temperature. The solvent combination used was chloroform-methyl-isobutylketone (4:1). The plate was spotted with each of the mycotoxins in a solution of chloroform-methanol (1:1) and allowed to develop. 14 cm from the spotting line in a tank saturated with the solvent vapour. The plate was removed from the chamber and dried at room temperature. The spots were detected by exposure to long wavelength (366 mμ) UV illumination and spraying with colour reagents.

The formation, in situ, of fluorescent derivatives can be used to a) detect non-fluorescent mycotoxins, b) enhance the fluorescence of naturally fluorescing mycotoxins and c) confirm the presence of presumptive mycotoxins.

The non-fluorescent trichothecenes may be detected by chemical derivatisation. T-2 toxin, for example, appears as a grey-blue fluorescent spot after spraying with 20 per cent concentrated sulphuric acid in methanol and heating at 110 °C for 3 to 4 minutes. Alternatively, T-2 toxin will afford a bright blue fluorescence if treated with a mixture of aluminium chloride (in water:ethanol, 1:1) and chromotropic acid (in concentrated sulphuric acid:water, 5:3) followed by heating at 110 °C.

The natural fluorescence of sterigmatocystin may be enhanced, to afford a bright yellow spot, by spraying with a 24 percent solution of aluminium chloride in 95 percent aqueous ethanol and heating at 105 °C for 10 minutes. The identity of sterigmatocystin may be confirmed by the formation of the acetate or hemiacetal derivatives. Similarly, the long-wave fluorescence of zearalenone and ochratoxin A may be enhanced if the plate is sprayed with aluminium chloride solution. The presence of ochratoxin A may be confirmed by the formation of the ethyl ester derivative. The natural, short-wave (254 nm) fluorescence of patulin can be enhanced by treatment with 0.5 percent aqueous 3-methyl-2-benzothiazolinone hydrazone (MBTH) followed by heating at 130 °C for 15 minutes. If penicillic acid is treated with MBTH, a visible pale yellow spot is produced. Citrinin tends to streak in many solvent systems and is probably best chromatographed on silica gel TLC plates impregnated with oxalic acid or ethylenediaminetetra-acetic acid (EDTA). The long-wave yellow fluorescence of citrinin may be converted to a green fluorescence by spraying the plate with 14 percent (w/w) boron trifluoride in ethanol. The presence of citrinin may be confirmed by the formation of the acetate derivative.

**Results and discussion.** The spray reagents used were: (a) Concentrated sulphuric acid. After spraying the plate was heated at ca. 110° for 10 min. (b) 1% ethanolic ferric chloride.

The colours of the various mycotoxins under UV light and after spraying with the colour reagents are recorded in Table 1. Also included in Table 1 are the fungal sources and the R<sub>f</sub> (x 100) values. The reported R<sub>f</sub> value for each mycotoxin is the average of ten independent determinations. It is apparent from the R<sub>f</sub> values that the mycotoxins are well resolved in this system.

Table 1 – The separation of mycotoxins on 0,25 mm layer of silica gel G impregnated with oxalic acid

Mycotoxin	Reference	Fungus	R <sub>f</sub> value (x 100)	Fluorescence	Colour reagents	
					H <sub>2</sub> SO <sub>4</sub>	FeCl <sub>3</sub>
Aspertoxin	8	A. flavus	12	light yellow	green-yellow	---
Ochratoxin B	9	A.ochraceus	20	blue	---	red-brown
Secalonic acid D	10	P. oxalicum	23	dark	light brown	light brown
8α-(3-methylbutyryloxy)- 4β, 15-diacetoxyscirp- -9-en-3α-ol	11	F.tricinclum	28	---	lead grey	---
Aflatoxin G1	12	A. flavus	30	green	green-grey	---
Aflatoxin B1	12	A. flavus	40	blue	green-grey	---
6β-Hydroxyroscnonolactone	13	F. roseum	44	---	orange-red	---
Ochratoxin A	9	A.ochraceus	48	green	---	red-brown
Cyclopiazonic acid	14	P.cyclopium	65	dark	red-brown	red-brown
Zearalenone	15	F.graminearum	72	faint blue	light yellow	red-brown
Sterigmatocystin	16	A.nidulans A.versicolor Bipolaris sp.	85	orange	green-grey	green

A suitable chromogenic reagent for these mycotoxins is a solution of one per cent ceric sulphate in 6 N sulphuric acid. Some compounds give a characteristic colour with a specific reagent, e.g. cyclopiazonic acid gives a violet colour on spraying with Ehrlich reagent. Cyclopiazonic acid also turns violet-red on prolonged standing on the silica gel plates impregnated with oxalic acid.

**Conclusions and prospect of further research.** If oxalic acid is omitted from the silica gel slurry, the mobility of the neutral metabolites are virtually unaffected, whereas the acidic compounds e.g. cyclopiazonic acid, secalonic acid D, and ochratoxins A and B do not move. This can be employed as a confirmation. Absolute confirmatory tests, e.g. by direct comparison with a standard reference sample, by physico-chemical methods or bio-assay [17] are essential for the final proof for the presence of a suspected mycotoxin in foodstuffs.

## СПИСОК ЛІТЕРАТУРИ

1. Forgacs J. Feedstuff / J. Forgacs // Appl. Microbiol – 2004. – Vol.34. – P. 124.
2. Nature / K.Sargeant, A.Sheridan, J.O'Kelly [et al.] // Med. Pregl. – 2009. – Vol. 192. – P. 1095.
3. American Academy of Pediatrics. Toxic effects of indoor molds. Pediatrics. – 2006. – Vol.101. – P. 712-714.
4. Anderson S. J. Compositional changes in surface mycoflora during ripening of naturally fermented sausages / S. J. Anderson // J. Food Protect. – 2005. – Vol.58. – P. 426-429.
5. Barrett J. Mycotoxins: of molds and maladies / J. Barrett // Environ. Health Perspect. – 2009. – Vol.108. – P. A20-A23.
6. Ochratoxin production by the *Aspergillus ochraceus* group and *Aspergillus alliaceus*. / P. Bayman, J. L. Baker, M. A. Doster [et al.] // Appl. Environ. Microbiol. – 2002. – Vol.68. – P. 2326-2329.
7. Beardall J. M. Disease in humans with mycotoxins as possible causes. / J. M. Beardall, J. D. Miller. – 2004. – P. 487-539.
8. Beasley V. R. Trichothecene mycotoxicosis: pathophysiologic effects / V. R. Beasley. – 2009. – Vol.1. – P. 10.
9. Belkin L. Haunted by mold / L. Belkin // New York Times Magazine. – 2001. – Vol.62. – P. 64-65.
10. Bennett J. W. Mycotoxins, mycotoxicoses, mycotoxicology and mycopathology / J.W. Bennett // Mycopathologia. – 2007. – Vol.100. – P.3-5.
11. Bennett J. W. Pride and prejudice: the story of ergot / J. W. Bennett, R. Bentley // Perspect. Biol. Med. – 1999. – Vol.42. – P. 333-355.
12. Bennett J. W. One gene to whole pathway: the role of norsolorinic acid in aflatoxin research. / J. W. Bennett, P.-K. Chang and D. Bhatnagar // Adv. Appl. Microbiol. – 2007. – Vol.45. – P. 1-15.
13. Bentley R. Constructing polyketides: from Collie to combinatorial biosynthesis. / R.Bentley and J. W. Bennett // Annu. Rev. Microbiol. – 1999. – Vol.53. – P. 411-446.
14. Berry C. L. The pathology of mycotoxins / C. L. Berry // J. Pathol. – 2008. – Vol. 154. – P.301-311.
15. Betina V. Mycotoxins: chemical, biological and environmental aspects / V. Betina. – 2009. – Vol.9. – P. 9.
16. Betina V. (ed.). 2004. Mycotoxins: production, isolation, separation, and purification / V. Betina // Elsevier, Amsterdam, The Netherlands.
17. Blanc P. J. Production of citrinin by various species of *Monascus* / P.J. Blanc, M.O. Loret, G. Goma // Biotechnol. Lett. – 2005. – Vol.17. – P. 291-294.

## REFERENCES

1. Forgacs J. Feedstuff / J. Forgacs // Appl. Microbiol – 2004. – Vol.34. – P. 124.
2. Nature / K.Sargeant, A.Sheridan, J.O'Kelly [et al.] // Med. Pregl. – 2009. – Vol. 192. – P. 1095.
3. American Academy of Pediatrics. Toxic effects of indoor molds. Pediatrics. – 2006. – Vol.101. – P. 712-714.
4. Anderson S. J. Compositional changes in surface mycoflora during ripening of naturally fermented sausages / S. J. Anderson // J. Food Protect. – 2005. – Vol.58. – P. 426-429.
5. Barrett J. Mycotoxins: of molds and maladies / J. Barrett // Environ. Health Perspect. – 2009. – Vol.108. – P. A20-A23.
6. Ochratoxin production by the *Aspergillus ochraceus* group and *Aspergillus alliaceus*. / P. Bayman, J. L. Baker, M. A. Doster [et al.] // Appl. Environ. Microbiol. – 2002. – Vol.68. – P. 2326-2329.
7. Beardall J. M. Disease in humans with mycotoxins as possible causes. / J. M. Beardall, J. D. Miller. – 2004. – P. 487-539.
8. Beasley V. R. Trichothecene mycotoxicosis: pathophysiologic effects / V. R. Beasley. – 2009. – Vol.1. – P. 10.
9. Belkin L. Haunted by mold / L. Belkin // New York Times Magazine. – 2001. – Vol.62. – P. 64-65.
10. Bennett J. W. Mycotoxins, mycotoxicoses, mycotoxicology and mycopathology / J.W. Bennett // Mycopathologia. – 2007. – Vol.100. – P.3-5.
11. Bennett J. W. Pride and prejudice: the story of ergot / J. W. Bennett, R. Bentley // Perspect. Biol. Med. – 1999. – Vol.42. – P. 333-355.
12. Bennett J. W. One gene to whole pathway: the role of norsolorinic acid in aflatoxin research. / J. W. Bennett, P.-K. Chang and D. Bhatnagar // Adv. Appl. Microbiol. – 2007. – Vol.45. – P. 1-15.
13. Bentley R. Constructing polyketides: from Collie to combinatorial biosynthesis. / R. Bentley and J. W. Bennett // Annu. Rev. Microbiol. – 1999. – Vol.53. – P. 411-446.
14. Berry C. L. The pathology of mycotoxins / C. L. Berry // J. Pathol. – 2008. – Vol. 154. – P.301-311.
15. Betina V. Mycotoxins: chemical, biological and environmental aspects / V. Betina. – 2009. – Vol.9. – P. 9.

16. Betina V. (ed.). 2004. Mycotoxins: production, isolation, separation, and purification / V. Betina // Elsevier, Amsterdam, The Netherlands.

17. Blanc P. J. Production of citrinin by various species of *Monascus* / P.J. Blanc, M.O. Loret, G. Goma // Biotechnol. Lett. – 2005. – Vol.17. – P. 291-294.

#### **Распределение и определение нескольких микотоксинов с помощью тонкослойной хроматографии**

**А.В. Билан**

В статье описано разработку новой системы для одновременного разделения и обнаружения одиннадцати различных микотоксинов. Водорастворимые и липидные балластные вещества удаляются из экстракта с помощью жидкостного раздела. Для смесей, содержащих кислые микотоксины, например циклопиазоновую кислоту, секалоновую D кислоту, охратоксины А и В, очистка достигается путем удаления нейтрального материала по стандартной методике. Для анализа афлатоксинов, охратоксина, зеараленона, цитринина, патулина, трихотеценов, циклопиазоновой кислоты, рубратоксина, стеригматоцистина, пеницилловой кислоты и цитреовиридина использовали ТЛХ. Для опыта использовали силикагель G (Merck), суспендированный в 0,4 N водном растворе щавелевой кислоты в соотношении (1:2). Совмещенным растворителем был хлороформ-метил-изобутилкетон (4:1). Пятна микотоксинов проявляли под УФ-светом с длиной волны (366 Нм) и распылением реагентов.

**Ключевые слова:** микотоксины, экстракты, очистка, тонкослойная хроматография, обнаружение.

#### **The separation and detection of several mycotoxins by thin-layer chromatography**

**A. Bilan**

The article described methods alternatively, T-2 toxin will afford a bright blue fluorescence if treated with a mixture of aluminium chloride (in water:ethanol, 1:1) and chromotropic acid (in concentrated sulphuric acid:water, 5:3) followed by heating at 110 °C. The natural fluorescence of sterigmatocystin may be enhanced, to afford a bright yellow spot, by spraying with a 24 percent solution of aluminium chloride in 95 percent aqueous ethanol and heating at 105 °C for 10 minutes. The identity of sterigmatocystin may be confirmed by the formation of the acetate or hemiacetal derivatives. Similarly, the long-wave fluorescence of zearalenone and ochratoxin A may be enhanced if the plate is sprayed with aluminium chloride solution. The presence of ochratoxin A may be confirmed by the formation of the ethyl ester derivative.

**Key words:** mycotoxins, extracts, cleaning, thin layer chromatography, detection.

*Надійшла 27.10.2015 р.*