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«TRAINING ACTIVITIES ON FOOD CONTAMINATION CONTROL AND MONITORING WITH SPECIAL REFERENCE TO MYCOTOXINS»

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CHEMICAL ANALYTIC METHODS OF TRICHOTHECENE MYCOTOXINS. BASIC FACTS ABOUT TRICHOTHECENES

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CHEMICAL ANALYTIC METHODS OF TRICHOTHECENE MYCOTOXINS. BASIC FACTS ABOUT TRICHOTHECENES.*

V.S. Sobolev

Introduction

Among the toxins of microscopic fungi a significant place is occupied by trichothecene mycotoxins (trichothecenes) representing a group of chemically related compounds - derivatives of the 12, 13-epoxytrichothe-9-cene. Trichothecene toxins are produced by various fungi species of the genera Fusarium, Myrothecium, Stachybotrys, Trichoderma, Cephalosporium, etc. They can contaminate food raw materials, foodstuffs and fodder. At present, the role of microscopic fungi - producers of trichothecenes - as an etiological factor of a whole range of alimentary toxicoses in man and animals, is quite clear. Numerous experiments show that independent trichothecene mycotoxins, or, which is more likely, their combinations, serve as causative agents of the above toxicoses.

The most common trichothecene-induced diseases include alimentary toxic aleukia (ATA), red-mold disease (akakabibyo toxicoses), stachybotryotoxicoses, dendrodochiotoxicoses, etc.

The first description of an alimentary disease in man and domestic animals - "intoxicating bread" - observed in the Ussuri kraj (Eastern Russia), and which was most likely caused by trichothecenes, dates back to 1891. In 1931 in the Ukraine and in Central Europe there was a mass equine disease

- stachybotryotoxicosis - induced by the fodder contaminated with the toxigenic fungus Stachybotrys alternans. A lethal hen's disease (1940-1946) was also induced by the fodder, contaminated with S. alternans. Repeated outbreaks of animal diseases with symptoms common for stachybotryotoxicoses were registered in Hungary. More recent investigations showed that stachybotryotoxicoses were caused by macrocyclic trichothecenes. In 1942-1947, and particularly in 1944, in the Orenburg oblast over 10% of the population consuming overwintered cereals, were affected by severe toxicosis - alimentary toxic aleukia. Most recently it was demonstrated that fungi Fusarium sporotrichiella inducing toxicoses, produced, inter alia, trichothecene mycotoxins.

Beginning from the 20-ies to the present time alimentary diseases of livestock and poultry with similar signs have been observed in various countries. Table 1 presents some of the above diseases.

Composition and chemical properties of trichothecenes

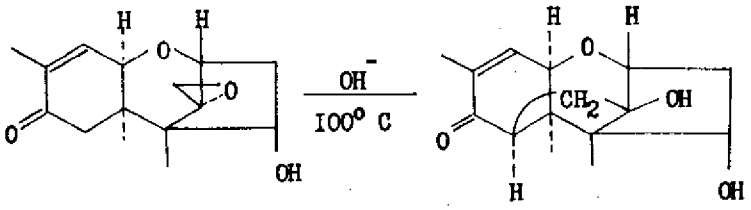
Trichothecenes form a family of structure-related sesquiterpenoids; the basis of the structure represents a system of rings called the trichothecane. All trichothecenes - natural metabolites - contain a double bond (C-9 - C-10) and an epoxy group at the 12-th and 13-th carbon atoms, and can be characterized as 12, 13-epoxytrichothecenes.

In nature trichothecenes are divided by their chemical properties into 4 groups: A, B, C and D (Tables 2-4). Group

B trichothecenes differ from those of Group A in the fact that the former contain a carbonyl group at C-8. The Group C includes macrocyclic trichothecenes. The only representative of the Group D is crotocin which contains an epoxy group at C-7-C-8.

Solid trichothecenes are resistant at storage and are rather long-term survivers in the form of solutions in aprotic solvents, especially, at low temperature. About 50% of trichothecene (nivalenol and deoxynivalenol) added to foodstuffs remain unchanged in the process of culinary treatment (baking at 210°C, roasting at 140°C, boiling).

Durable water boiling results in hydration of some trichothecenes. In solutions at extremal pH values trichothecenes undergo the following changes: ester groups are saponified by alkali, while the epoxy group (C-12 - C-13) is open up under the effect of potent mineral acids. Under the effect of alkali at elevated temperature 8-keto-trichothecenes are transformed into the corresponding derivatives:



In general, trichothecenes enter in the majority of reactions characteristic of the functional groups included

into their composition.

The interrelation of trichothecene toxins' composition with their biological activity is quite interesting. Thus, partial or total saponification of trichothecenes to the corresponding alcohols usually results in decreased toxicity. The reduction of the double bond C-9 - C-10 somewhat reduces the toxicity level. The compounds with an open epoxy group C-12 - C-13 are practically totally nontoxic.

Producers of trichothecenes

In contrast to other mycotoxin groups trichothecenes are produced by dozens of species of microscopic fungi of various genera. The overwhelming majority of trichothecene-producing strains is generated by several toxins. For example, Fusarium sporotrichiella produces T-2, HT-2, NT-1 toxins, neosolaniol, T-2 triol. Table 5 contains data on the production of trichothecenes by some Fusarium species.

Depending on cultivation conditions, one and the same fungi strain can produce various trichothecenes in different proportions (Table 6).

Occurrence of trichothecenes

Trichothecene-producing fungi are common in nature. A certain regularity in the occurrence of toxigenic trichothecene-producing fungi by climatic zones should be noted. Thus, F. sporotrichioides and F. pose, producing Group A trichothe-

cenes, are mainly observed in areas with low mean annual temperature (USSR, Nothern Europe, Canada), while F. graminearum, producer of Group B trichothecenes, is common in warm territories (Southern parts of the USA).

It is important to note that by the present time, about 50 various trichothecenes - fungi metabolites, have been discovered, however, only four of them have been identified as natural contaminants of foodstuffs: T-2 toxin, diacetoxiscirpenol, nivalenol and deoxynivalenol (vomitoxin).

There are few literature data about trichothecene contamination of foodstuffs and fodder. This is primarily explained by the fact that simple and reliable methods of trichothecene analysis have not been developed till very recently. While analyzing the data about trichothecene contamination of foodstuffs one can draw a conclusion that mainly corn and barley, and also combined fodder, are subject to contamination. In such cases deoxinivalenol (vomitoxin) serves as basic contaminant. It should be noted that it is those lots of foodstuffs were subject to the analysis that manifested any toxicity at their feeding to animals or when the latter refused to consume the fodder from such lots. In the overwhelming majority of cases the maximal trichothecene concentration in food raw materials and fodder did not exceed 15-20 mg/kg.

Toxic properties of trichothecenes

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All trichothecene mycotoxins represent highly toxic com-

pounds for such experimental animals as dogs, cats, rats, mice, guinea pigs, etc. Basically, the most common signs of contamination are: vomiting, neasea, diarrhea, hyperemia of the gastro-intestinal tract mucosa, leukopenia. It is characteristic of trichothecenes to induce inflammatory reactions at their contact with the skin of experimental animals. Animal rejection of fodder represents a characteristic syndrome of fusariotoxins. Some publications contain abortion cases in swine and growth retardation in the young. Table 7 contains data about toxic properties of trichothecenes. It is significant to mention that trichothecenes possess antibiotic, phytotoxic and cytotoxic properties (particularly related to fissionable cells). Trichothecene toxicity depends on their nature, species and age of experimental animals, and other factors. In general, young animals are more sensitive to trichothecenes, on the other hand, the toxicity does not greatly depend on animal sex and on the mode of toxin injection. T-2 toxin, and also macrocyclic trichothecenes are most toxic.

In view of the fact that trichothecene-producing fungi are known to produce other micotoxins, e.g. zearalenone, their combined toxicity in real assessment of toxicity of trichothecene-contaminated products, should be considered.

At present, the mechanism of trichothecene toxic effect and ways of their organic metabolism have not been adequately studied. The transformation of T-2 toxin into HT-2 toxin and fusarenon-X into nivalenol in the liver of animals can serve as example of a well studied metabolic process; thus formed diacetyl metabolites are excreted with urine and feces.

Biological methods of trichothecene analysis

High trichothecene toxicity permits the use of biological methods for the identification of trichothecenes in various substrates. Thus, larvae Artemia salina, some species of aquarium fishes, several higher plants, etc., are frequently used for the identification of trichothecenes. Of interest is the "autobiographic technique" involving the use of the yeast culture Candida pseudotropicalis 44 nk, which is sensitive to trichothecenes. The technique consists in the application of an agar yeast culture suspension on a preeluted chromatographic plate containing trichothecenes, and its thermostat treatment in optimal conditions ending in the appearance of mycelium. Mycelium does not grow in areas of trichothecene presence, while the area of a thus developed "bare" spot is taken for the identification of trichothecene volume. The sensitivity of the above technique is up to 25-50 ng of T-2 toxin per spot.

Chemical methods of trichothecene analysis

The majority of analytic methods applied for Groups A and B trichothecenes, contained in various substrates, usually include the following stages: extraction, purification, identification and quantitation.

a) Extraction of a trichothecene fraction

Aquaous methanol, chloroform, ethyl acetate and aceto-

nitril are most frequently used for trichothecene extraction. Group A trichothecenes are most well soluble in aprotic solvents like chloroform, ethyl acetate, acetone, while Group B trichothecenes are best soluble in polar solvents: alcohols, acetonitril and water. Many Group B trichothecenes can be extracted from substrate using the solvents for Group A trichothecenes. Aqueous methanol with various combinations of original components, characterized by highest selectivity and adequate reproducibility of results, has found most common application in the extraction of different trichothecenes. For example, ethyl acetate (compared with aqueous methanol), while being an effective solvent in the extraction of Group A trichothecenes, turns out to be considerably less selective, which requires a more thorough purification procedure.

b) Extract purification

The majority of described purification techniques involve the following traditional stages: liquid-liquid partition, column chromatography on silica gel, preparative thin-layer chromatography (TLC), etc. As a rule, the identification and quantitation of trichothecenes require more than two of the above mentioned manipulations. For example, a number of purification techniques of aqueous methanol extracts include hexane-defatting with the following reextraction of trichothecenes into chloroform. In other techniques an aqueous methanol extract is evapourated in vacuum, while the residue is purified by column chromatography or preparative TLC. In the overwhelming majority of cases the liquid-liquid purifi-

cation is followed by the silica gel purification. Basically, the elution systems include: chloroform/methanol (from 97:3 to 90:10), chloroform/acetone (from 90:10 to 60:40), toluene/ethyl acetate (67:33), and others. Other adsorbents, like florisil, sephadex, etc., are very rarely used in the purification of extracts. Recently, the reversed phase column chromatography cleanup (Sep Pak C_{18} - catridges) has been quite frequently utilized at the last purification stage. This facilitates the procedure of purification and reduces time losses. Among other analytic techniques, the one involving the use of dialysis, seems interesting, however, rather time-consuming. In this technique acetonitril, which is used for the extraction of an analyzed sample, is subject to dialysis against aqueous acetone (70:30). Toxins are reextracted from aqueous acetone into chloroform, and then detected by means of TLC.

c) Identification and quantitation of trichothecenes

While trichothecenes possess rather similar chromatographic properties, they can be separated and purified from undesirable admixtures using the TLC technique on silica gel, and more rarely, on aluminium oxide. The advantages of the TLC technique consist in its simplicity, availability, efficiency and possibility of using specific spray reagents, to a certain degree confirming the relationship of a substance to a trichothecene group. The major TLC drawback lies in its rather high detection limit compared with that of gas-liquid chromatography (GLC) and other instrumental analytic techniques.

Teble 8 presents solvent systems and values of chromatographic mobility (R_f) of some trichothecenes which are most frequently used in TLC.

Trichothecenes do not possess the capability of fluorescence or absorption of visible or ultraviolet (UV) light (except for several macrocyclic Group C trichothecenes), thus, their spectral identification presents certain difficulties.

A rather sensitive and specific technique of developing Group A trichothecene spots consists in the interaction of the latter with sulfuric acid; a plate is sprayed with a 15% methanol sulfuric acid solution with the following heating at 100-110°C. Trichothecene spots are charred, while long-wave UV light displays light-blue fluorescence; the detection limit is up to 0.1-0.2 µg per spot. Group B trichothecenes do not possess this property. On the contrary, aluminium chloride induces blue fluorescence only in Group B trichothecenes. The plates are sprayed with 50% methanolic aluminium chloride solution and are heated at 130°C during 10 min., and then are studied in long-wave UV light (365nm). The above reaction was used as basis for the development of a more sensitive fluorescent technique of fusarenon-X identification: up to 50 ng per spot. A rather universal, however, not quite specific technique consists in the visualization of trichothecenes using p-anise aldehyde. After spraying the plates are dried and heated at 100-120°C during 10 min, which results in the coloring of Group A trichothecenes (and frequently also of the components of an analyzed extract) in various shades of pink-violet color, while Group B tricho-

thecenes aquire a coloring from yellow to brown.

When a color reaction of trichothecenes with 4-(p-nitrobenzyl) pyridine (NBP) is used all the above toxins are identified within the range from 0.02 μg per spot. The plates are treated with 3% NBP solution followed by heating at 150°C during 30 min., and then with 10% solution of tetraethylenepentamine. After this treatment all trichothecene spots turn blue. This spray reagent is specific for 12, 13-epoxy group.

Of interest is the fluorescence technique of trichothecene identification using nicotinamide and 2-acetylpyridine. The reaction results in the formation of stable fluorescent derivatives of naphthyledine; the detection limit is 20-25 ng per spot.

The most sensitive noninstrumental technique is that of fusarenon-X identification, based on fusarenon-X reaction with $ZrO(NO_3)_2$ in the presence of ethylene diamine resulting in the formation of a fluorescent adduct. The technique permits to identify fusarenon-X in a sample with the level of 25 μg/kg.

For the identification of diacetoxyscirpenol and other related compounds on TLC plates the Ehrlich spray reagent (p-dimethylaminobenzaldehyde) can be used, the reaction of which with the toxin results in violet coloring. No such coloring is observed with T-2 toxin; in this way diacetoxyscirpenol, having a similar to T-2 R_f value, can be identified. For the identification of diacetoxyscirpenol phosphomolybdic acid can also be utilized, the 10% solution of which is taken for plate spraying; the reagent forms with the toxin a dirty-

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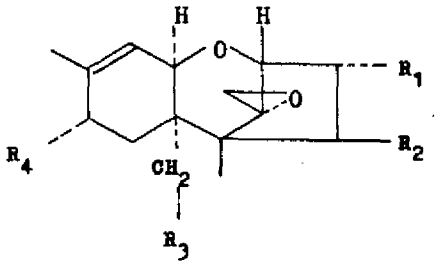
blue spot. This toxin is also identified as a result of the spraying with a chloroform solution of trichloride of antimony which brings about a purple-blue spot.

All color reactions for trichothecenes represent most simple and available techniques of identification and quantitation of the above mycotoxins, however, their specificity and sensitivity are considerably inferior to those of instrumental analytic techniques: gas-liquid chromatography (GLC) and chrometographic mass spectrometry. GLC on glass capillary columns is the most convenient technique of qualitative and quantitative identification of trichothecenes. This technique permits total separation of practically all Groups A and B trichothecenes in the form of their volatile derivatives; the detection limit is up to 50-100 µg/kg. The technique requires special equipment and higher standards of work, compared to those of TLC, which makes it difficult to be used at routine screening. Chromatographic mass spectrometry gives direct information about the content of various trichothecenes in an analyzed sample, however, the above technique cannot be applied for routine analyses in view of high cost of the equipment; the detection limit of the technique is up to 1-5 µg/kg. The high-performance liquid chromatography (HPLC) finds still greater use in the analysis of trichothecenes, despite its difficulties of detection and lower sensitivity than that observed in GLC; with the use of a refractometric detector its detection limit raises up to 100-200 µg/kg.

Table 1. Alimentary toxicoses, their basic symptoms and species of fungi inducing the above diseases.

Toxicosis	Country, region	Affected	Fungus species	Symptoms
Intoxicating bread	USSR, Siberia	Man, horse, swine, poultry	G. saubinetti	Headache, rigor, vomiting
Red mold disease	Japan	Man, horse, swine, cow	F. graminearum	Nausea, vomiting, hemorrhage, feed rejection
Mold corn contamination	USA	Swine, cow	F. tricinctum	Feed rejection, vomiting
Alimentary toxic aleukia	USSR	Man, domestic animals	F. sporotrichioides	Nausea, vomiting, diarrhea, leukopenia, septic angina, hemorrhage
Stachybotryotoxicosis	USSR	Horse	Stachybotrys atra	Shock, dermatitic necroses, leukopenia
Red mold disease (bean husk contamination)	Japan	Horse	F. solani etc.	Convultions, round movements
Dendrodochiotoxicosis	USSR	Sheep, swine	Dendrochium toxicum	Inflammations, hemorrhage

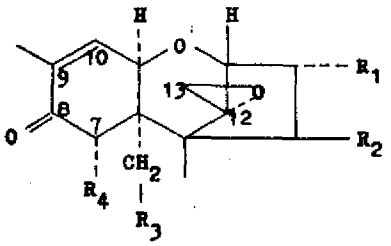
Table 2. Structure of some trichothecenes. Group A.



Toxin	R_1	R_2	R_3	R_4
Trichothecene	H	H	H	H
Trichodermol (Roridin C)	H	OH	H	H
Trichodermin	H	OAc*	H	H
Verrucarol	H	OH	OH	H
Scirpentriol	OH	OH	OH	H
T-2 tetraol	OH	OH	OH	OH
Monoacetoxyscirpenol	OH	OH	OAc	H
Diacetoxyscirpenol	OH	OAc	OAc	H
Neosolaniol	OH	OAc	OAc	OH
T-2 toxin	OH	OAc	OAc	$OCOCH_2CH(CH_3)_2$
HT-2 toxin	OH	OH	OAc	$OCOCH_2CH(CH_3)_2$
Acetyl-T-2 toxin	OAc	OAc	OAc	$OCOCH_2CH(CH_3)_2$

* - OAc = CH_3CO-

Table 3. Structure of some trichothecenes. Group B



Toxin	R_1	R_2	R_3	R_4
Nivalenol	OH	OH	OH	OH
Fusarenon-X	OH	OAc	OH	OH
Deoxynivalenol	OH	H	OH	OH
Diacetylnivalenol	OH	OAc	OAc	OH
Trichothecine	H	$OCOCH=CHCH_3H$	H	H

Structure of crotocin trichothecene - natural metabolite. Group D.

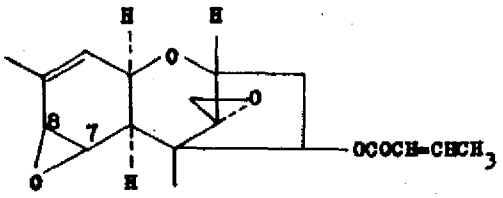


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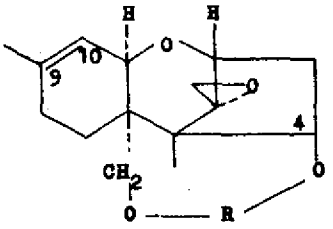
Table 4. Structure of some trichothecenes. Group C.



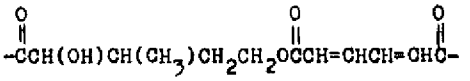
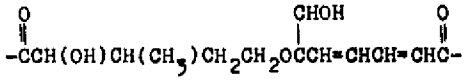
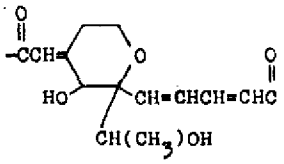
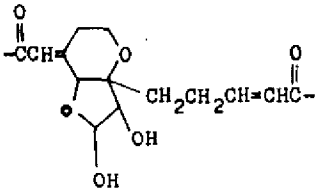
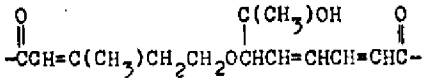
Toxin	R
Verrucarin A	
Roridin A	
Satratoxin H	
Vertieporin	
Roridin E	

Table 5. Production of some trichothecenes by various *Fusarium* species.

Fungus species	Trichothecene toxins[+/]					
	Type A			Type B		
	T-2	NS	DAS	NV	FX	DON
F. tricinctum	+	+	+			
F. sporotrichioides	+	+	+			
F. poae	+	+	+			
F. acuminatum	+	+				
F. graminearum				+	+	+
F. nivale				+	+	
F. lateritium		+	+	+		
F. equiseti		+	+	+	+	
F. semitectum		+	+	+	+	

[+/] T-2 = T-2 toxin
NS = Neosolaniol
DAS = Diacetoxyscirpenol
NY = Nivalenol
FX = Fusarenon-X
DON = Deoxynivalenol (vomitoxin)

Table 6. Cultivation-related trichothecene production by *Fusarium* fungi

Culture	*Fusarium tricinctum*		
Temperature	Low	⟶	High
Cultivation time	Short	⟶	Long
Medium	Liquid	⟶	Solid
Produced toxin	T-2 toxin	⟶	HT-2 toxin

Culture	*Fusarium nivale*				
Temperature	Low	⟶	High		
Cultivation time	Short	⟶	Long		
Medium	Liquid	⟶	Solid		
Produced toxin	Diacetyl-nivalenol	⟶	Monoacetyl-nivalenol	⟶	Nivalenol

Table 7. Comparison of toxin properties of some trichothecenes

Toxin	LD_{50}		Nausea inducing dose		Dose inducing a positive skin test
	Mice	Chick embryo	Ducklings	Cats	Guinea-pigs
	mg/kg ip[+/]	µg/egg		mg/kg sc[+/]	µg
(A)					
T-2 toxin	5.2	0.07	0.1	0.1	0.2
HT-2 toxin	9.0	0.5	0.1		0.2
Diacetoxy-scirpenol	23	0.09	0.2		0.2
Neosolaniol	14.5	5.0	0.1		1.0
(B)					
Nivalenol	4.1	4.0	1.0		10
Fusarenon-X	3.3	2.6	0.4	1.0	1.0
Deoxynivalenol	70		13.5		-
(C)					
Verrucarin A	1.5 (iv[+/])				0.05
Roridin A	1.0 (iv)				0.05

[+/] ip = intraperitoneally
sc = subcutaneously
iv = intravenously

Table 8. Thin-layer chromatographic mobility of some trichothecenes (R_f values)

Trichothecene	Solvent systems											
	1	2	3	4	5	6	7	8	9	10	11	12
(A)												
Trichodermol							0.17					
Trichodermin							0.51					
Verrucarol							0.03					
Scirpentriol			0.036					0.071				
Monoacetoxy-scirpenol			0.066					0.121				
Diacetoxy-scipenol			0.474		0.52	0.50	0.14	0.373	0.47	0.46	0.68	0.82
T-2 tetraol			0.00				0.00	0.021				
Neosolaniol			0.188		0.38	0.29		0.152	0.15	0.25	0.32	
HT-2 toxin			0.101		0.30	0.21	0.03	0.125	0.10	0.17	0.23	
T-2 toxin			0.528		0.55	0.52	0.16	0.41	0.61	0.68	0.78	0.81
(B)												
Trichothecolon							0.13					
Trichothecin							0.53					0.815
Nivalenol				0.22	0.09	0.03			0.02	0.01	0.04	0.675

Table 8.(Cont.) Thin-layer chromatographic mobility of some trichothecenes (R_f values)

Trichothecene	Solvent systems											
	1	2	3	4	5	6	7	8	9	10	11	12
Fusarenon-X			0.17	0.64	0.41	0.29		0.25	0.31	0.21	0.32	
Diacetyl-nivalenol					0.51	0.44			0.47	0.43	0.54	
Tetraacetyl-nivalenol					0.62	0.63						
Deoxynivalenol (C)			0.066	0.47	0.31	0.20		0.157				
Verrucarin A	0.28	0.59					0.47					
Verrucarin B	0.47	0.69					0.63					
Verrucarin	0.59						0.64					
Roridin A	0.18	0.21					0.20					
Roridin 1 (D)	0.35						0.29					
Crotocol							0.07					
Crotocin					0.59	0.65	0.34					

+/1 - chloroform/methanol (98:2)
2 - chloroform/methanol (97:3)
3 - chloroform/methanol (95:5)
4 - chloroform/methanol (7:1)
5 - benzene/acetone (1:1)
6 - chloroform/acetone (3:2)
7 - benzene/tetrahydrofuran (85:15)
8 - ethyl acetate/toluene (3:1)
9 - ethyl acetate/hexane (3:1)
10 - ethyl acetate of chloroform and propanol (95:5:5)
11 - ethyl acetate of chloroform and ethanol (90:5:5)
12 - n-butanolacetic acid/water (4:1:1, upper layer)

"CHEMICAL METHODS FOR THE IDENTIFICATION OF TRICHOTHECENE MYCOTOXINS (VOMITOXIN)".

Introduction

Recently, the interest to vomitoxin as one of basic contaminants (trichothecene group) of foodstuffs and food raw materials, has grown considerably. For example, in Canada (Ontario) a considerable part of the whole 1980 corn yield was contaminated with vomitoxin. The level of vomitoxin contamination of a great number of studied samples constituted 1 mg/kg. An elevated level of this toxin in fodder brings about a considerable economic damage in the form of livestock murrain, fodder rejection by livestock and decreased animal weight. Thus, feeding swine with vomitoxin-contaminated grain on the level of 0.3-0.7 mg/kg resulted in the decreased fodder intake and lower animal weight.

Only several countries of the world adopted the documents standardizing the vomitoxin content in human foodstuffs. The MPC (maximal permissible concentration) adopted in the USA for grain produce vomitoxin constitutes 2000 µg/kg (2 mg/kg), while in the USSR the above value for the same produce is 1000 µg/kg (1 mg/kg).

A sufficiently reliable and available vomitoxin identification technique in foodstuffs (cereals) and fodder (combined fodder) is the TLC analysis with use of specific spray reagents: p-anise aldehyde, aluminium chloride, 4-(p-nitro-

benzyl)-pyridine, etc.

Purpose of the seminar

Aquaintenance of the students with one of the simplest techniques of vomitoxin detection in grain produce and combined fodder and practical mastering of the above technique (together with highly efficient and precise GLC and HPLC techniques).

SAMPLE ANALYSIS FOR VOMITOXIN: PREPARATORY MANIPULATIONS

1. Preparation of a standard vomitoxin solution

A solution with the vomitoxin concentration of 0.5 μg/μl is prepared by means of weighing +/ of 5 mg of the toxin on electronic analytic scales with the precision up to 10^{-5} - 10^{-4}g, which is then placed in a 10 ml pycnometer and is dissolved in a mixture of chloroform and acetonitrile (9:1). The solution is stored in a refrigerator.

2. Preparation of a spray reagent on the basis of p-anise aldehyde

0.5 ml of p-anise aldehyde is dissolved in 45 ml of

+/ The weighing is convenient to perform using a preweighed pan with the diameter of 4-6 mm made out of a thin aluminium foil. The weighed toxin is transfered into a pycnometer together with the pan.

methanol while another vessel is taken for a careful solution of 10 ml of ice-cold acetic acid and 5 ml of concentrated sulfuric acid in 40 ml of methanol. The prepared solutions are poured together and are thoroughly mixed. Fresh reagent is preferable.

3. Preperation of a spray reagent on the basis of aluminium chloride

An approximately 20% methanol solution of aluminium chloride is prepared; technical scales are used for the weighing of 20 g of aluminium chloride to be later dissolved in 80 ml of methanol.

4. Preparation of a No. 1 system of solvents for TLC

60 ml of chloroform with 40 ml of acetone are mixed in a 100 ml volumetric flask.

5. Preparation of a No. 2 system of solvents for TLC

80 ml of hexane and 20 ml of iso-propyl alcohol are mixed a 100 ml measuring flask.

ANALYSIS OF CEREALS AND COMBINED FODDER FOR VOMITOXIN

Sampling and sample preparation

An analyzed sample should be selected according to the sampling norms adopted in a number of countries. The sample should be representative; in no case the sample mass should be less than 1 kg. The selected sample is ground to flour in a

high-speed blender or in a lab grinder avoiding considerable heating of the sample. If by any reason it is impossible to grind a sample of 1 kg and over, in the exeptional case one can grind a sample weighing several dozens grams after a preliminery averefing of the sample.

Extraction

20 g of ground sample[+/] is weighed using technical scales with the precision of up to 0.1 g. Then the sample is placed in a 200-250 ml sealed conical flask and 100 ml of 50% aqueous methanol is added. The flask is shaken during 30 min in a shaker or the contents is mixed in a magnetic mixer. The mass is then filtered through a folded paper filter with a small volume (3-7 g) of Celite 545. Then a 60-70 ml aliquot is selected.

Purification by liquid-liquid extraction

The filtrate is defatted using 20-25 ml of hexane (or isooctane) in a 150-250 ml conical separation funnel. After the funnel shaking and phases separation the upper (hexane) layer is removed and 20-25 ml of chloroform is added to the remaining aqueous methanol layer. The funnel is shaken and

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[+/]During the technique mastering and verification of several analytic stages vomitoxin in the volume of 500 µg/kg is introduced into the studied sample by means of adding 20 µl of the standard solution with the concentration of 0.5 µg/ µl to the ground sample.

after the separation of the liquids the lower (chloroform) layer is transfered into a conical 100 ml flask. Chloroform is then repeated twice adding 5-8 ml of methanol into the separatory funnel, because in the process of extraction methanol partially turns into chloroform, which can result in the formation of a hardly separating emulsion. Combined chloroform extracts are dryed by anhydrous sodium sulfate during 15 min and then are filtered through a cotton ball placed in a chemical funnel. The filtrate is reduced at 40-50°C on a rotatory evaporator to the volume of 2-3 ml (solution A).

Chromatographic purification on silica gel

For the chromatographic purification of a vomitoxin fraction a small cotton ball is plaeed on the bottom of a glass chromatographic column with the diameter of 15-18 mm and 300-400 mm long. Then a chloroform suspension of 5 g silica gel (for column chromatography with 100-160 μm particles) is added; wall-adhered silica gel is washed down with small amounts of chloroform. The silica gel is allowed to sediment completely, another 10-15 ml of chloroform is poured in and, not allowing the solvent to drain down, 3-4 g of anhydrous sodium sulfate is injected into the column. When the chloroform level will touch the upper layer of sodium sulfate the A solution is pipeted into the column; the flask is rinsed with 2-3 ml of chloroform and the chloroform solution is also poured into the column. The column is eluted with 100 ml of chloroform, the chloroform eluate is wasted and the vomitoxin-containing fraction is eluted from the column, using 150 ml

of a chloroform/acetone mixture (3:2). The eluate is taken into a pear-shaped 250 ml flask and is reduced on a rotatory evaporator to the volume of 1-2 ml. The residue is transfered into a 8-12 ml flask with a conical bottom, while the walls of the 150 ml flask are twice rinsed with 2-3 ml of chloroform or acetone and the contents are transfered into the same (8-12 ml) flask and are evaporated to dryness using a rotatory evaporator. The residue is dissolved in a 100-150 µl mixture of chloroform and acetone (3:2). The flask is sealed avoiding evaporation of the solvent (solution B).

TLC-separation, detection and quantitetion of vomitoxin

For a two-dimentional TLC two precoated silica gel plates+/ are marked by a soft pencil as shown in Fig. 1. After marking the plates 10-20 µl of purified extract (solution B) is applied by means of a 25 µl microsyringe into the right bottom corner. Upper right and bottom left corners are used for the application of 4, 8, 12 and 8 µl of the standard vomitoxin solution (with the concentration of 0.5 µg/ µl), accordingly.++/ The diameter of applied spots should not exceed 3-4 mm. After the application of all spots, the plates are placed into a chromatographic chamber with the No. 1 sys-

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+/ Two plates are used for greater reliability of vomitoxin detection.

++/ Warm air flow, for example, generated by an electric hair dryer can speed up the procedure of spots application.

tem of solvents for the solvent level to be 7-10 mm inferior to that of applied sopts, while the plates are eluted till the marking line is reached. After the elution in the first direction the plates are extracted from the chamber, are dryed in a desiccator and are placed in a chamber with a No. 2 system of solvents +/ for the plates' development in in the second direction, preliminary turning the plates 90° clockwise. After the chromatographic treatment the plates are dryed in a dieseccator. One of the plates is sprayed with p-anise aldehyde solution and is directly placed in a desiccator, where it is kept for 3-5 minutes at a temperature of 110°C. In case vomitoxin is present in a studied sample the spot corresponding by color to those of standards should appear at the crosslines drawn parallel to the plate sides through the standard spot centres. The second plate is sprayed with methanol solution of aluminium chloride and is placed in a desiccator for 10 minutes at a temperature of 110-130°C. Positive vomitoxin reaction results in the appearance of a spot inducing blue fluorescence in long-wave UV light (365 nm) corresponding by R_f and color of fluorescence to standard spots. Comparing the fluorescence intensity of

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+/ One of the plates can be eluted in other solvent systems when another plate is eluted, e.g. the first direction: ethyl acetate/toluene 80:20, second direction: chloroform/methanol/water 90:9:1. Similar vomitoxin R_f values in the studied sample and standard spots on every plate serve as an additional proof of vomitoxin presence.

various amounts of the toxin standards with that of a corresponding extract spot (one of the standard spots, which better than others, corresponds to an extract vomitoxin spot by its intensity and area, is chosen), the amount of vomitoxin microgrammes per extract spot is determined. If the fluorescence intensity of an extract vomitoxin spot is higher then that of a standard spot, corresponding to 12/ul of the standard solution, a lower extract volume (solution B) should be applied onto the plate, or the solution should be diluted 2, 4 and more times and a repeated analysis should be performed.

The vomitoxin content in a sample is determined by the formula:

$$C = \frac{V_1 : V_2 \cdot m}{V_3 \cdot V_4 \cdot M} \text{ /ug/kg, where}$$

V_1 - aqueous methanol volume (ml) (100 ml);

V_2 - volume of the purified extract solution prior to thin-layer chromatography (/ul) (100-150/ul);

V_3 - volume of aqueous methanol extract, taken for the analysis (ml) (60-70 ml);

V_4 - volume of the extract solution applied

onto a plate (/ul) (10-20/ul);

M - analyzed sample mass (kg) (20 g = 0.02 kg);

m - vomitoxin amount per extract spot (/ug).

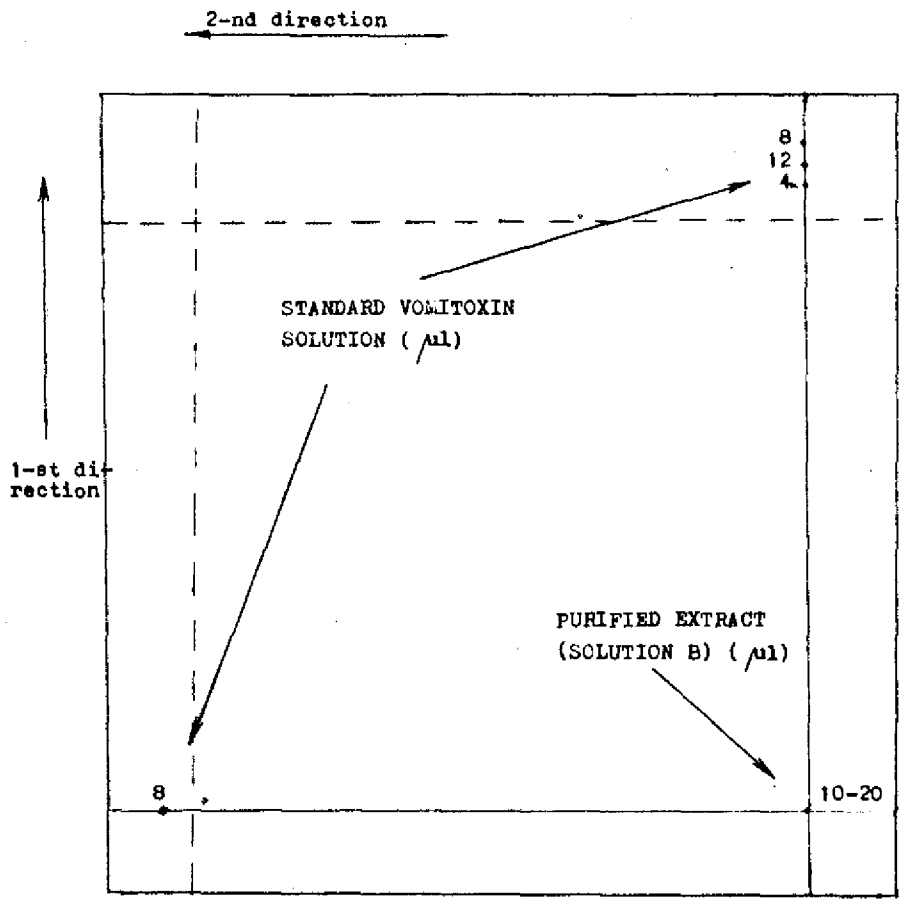

Fig. 1.

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