CHEMICAL AND BIOLOGICAL DETECTION OF 12,13-EPOXYTRICHOTHECENES ISOLATED FROM *FUSARIUM* SPECIES

T. TATSUNO[a], K. OHTSUBO[b] and M. SAITO[b]

(a) *The Institute of Physical and Chemical Research, Hirosawa, Wako-shi, Saitama-351, Japan*
(b) *The Institute of Medical Science, University of Tokyo, Shirokanedai, Minato-ku, Tokyo-108, Japan*

ABSTRACT

Our research team has isolated nivalenol, fusarenon-X, diacetylnivalenol, neosolaniol, 4-deoxynivalenol and 4-deoxy-3-acetylnivalenol from strains of *Fusarium* collected from rice, barley, cereals and pasture.

The 12,13-epoxytrichothecenes, after trimethylsilylation of the trichothecene compounds, can be chemically detected to the order of 1 μg ml^{-1} using gas chromatography and mass spectroscopy. A biological assay method using HeLa cells, including morphological mode of cell damage and also biochemical disturbances in not only protein synthesis but also DNA synthesis using radioactive precursors on the same cell culture system, is also presented.

Our research team has been involved with the toxicological study of *Fusarium* toxicoses ever since 1963, when the 1955 outbreak of food poisoning in Hoya City, Tokyo was shown to be caused by contamination of fungi of *Fusaria*[1]. We have so far isolated nivalenol[2], fusarenon-X[3] and diacetylnivalenol[4] as metabolic toxins of *Fusarium nivale*, neosolaniol[5,6] as that of *Fusarium solani*, and 4-deoxynivalenol and its 3-acetate as metabolic toxins of *Fusarium roseum*[7]. Compound-Q (12,13-epoxytrichothecene) and -R were isolated by Nozoe[8] and his co-workers from *Trichothecium roseum* in their biosynthetic study. These compounds have a common chemical structure, namely 12,13-epoxytrichothecene, and differ in side chains. Among 12,13-epoxytrichothecenes, we already know of the verrucarins[9], trichothecin[10] and trichodermin[11] as antifungal antibiotics and diacetoxyscirpenol[12] as a phytotoxic substance.

In experimental animals all of these compounds caused necrosis of the actively dividing cells in tissues such as the epithelium of the gastro-intestinal tract, especially of the small intestine, germ centre of the lymph follicles in the spleen, lymph nodes and other lymph apparatus, thymus and the bone

marrow[13, 14]. Furthermore, most of them showed a skin irritating or necrotizing effect on various kinds of animals[15]. The third column of *Table 1* shows the half-lethal doses in mice for the representative compounds.

As we have shown in our recent work[16] cytotoxicity to cultured cells can also be used as a sensitive screening test for mycotoxins, especially for ones that damage proliferating cells of the animals. We carried out this method using the plastic panel technique developed by Toplin[17]. The cells were incubated with test materials for 3 days with graded concentrations. The damage to the cells was arbitrarily graded[17] from 0, or no growth inhibition, to 4, or complete cytolysis. As is shown in the fourth column of *Table 1* all of the isolated 12,13-epoxytrichothecenes tested were growth inhibitory with doses of 0.1 to 10 μg ml^{-1}.

Table 1. Comparative toxicity of the trichothecenes isolated in Japan

Trichothecenes	Fungal strains from which the trichothecenes were isolated*	Acute toxicity to male mice (LD_{50} mg kg^{-1} i.p.)	Cytotoxicity to HeLa cells at 3.2 and 0.32 μg ml^{-1}		Inhibition of protein synthesis in rabbit reticulocytes[6, 21] (ID_{50}, μg ml^{-1})
Nivalenol	*F. nivale* (Fn 2B)	4.0	4	2	2.5
Fusarenon-X	*F. nivale* (Fn 2B), *F. epishaeria* (Fn M) *Gibberella zeae* (Ohita-II)	3.4	4	3	0.25
Diacetylnivalenol	*F. nivale* (Fn 2B)	3.5			0.30
Diacetoxyscirpenol	*F. solani* (M-1-1)	23.0			0.03
Neosolaniol	*F. solani* (M-1-1)	14.5			0.25
4-Deoxynivalenol	*F. roseum*				
3-Acetyl-4-deoxynivalenol	*F. roseum*				
Compound Q	*F. roseum*		1	0	
Compound R	*T. roseum*		3.5	1	
T-2 toxin	*F. solani* (M-1-1)	5.2	4	3	0.03
Trichothecin	*T. roseum*		4	2	0.30

* *F., Fusarium*; *T., Trichothecium*

The mechanism of action has been studied in detail with nivalenol and fusarenon-X using cultured cells[18–20]. They markedly inhibit protein and DNA synthesis of intact cells at doses of 0.3 to 1.0 μg ml^{-1}. The primary effect of fusarenon-X is a rapid and complete disaggregation of polyribosomes into monomers[20].

12,13-Epoxytrichothecenes strongly inhibit the protein-synthesizing function of rabbit reticulocytes[21]. As shown in the last column of *Table 1*, ^{14}C-leucine incorporation into the acid-insoluble fraction of rabbit reticulocytes is inhibited by 50 per cent at doses of 0.03 to 2.5 μg ml^{-1} of these substances.

In contrast mycotoxins other than 12,13-epoxytrichothecenes are quite insensitive to this test method[21]: no inhibition of protein synthesis of rabbit reticulocytes was observed with cyclochlorotine, aflatoxin B, butenolide and sporidesmin at doses of 50 μg ml^{-1}. It is possible, therefore, to utilize this as a specific bio-assay method for 12,13-epoxytrichothecene compounds.

The 12,13-epoxytrichothecenes in *Table 1* are produced by fungi which have a very close relationship with our foodstuff or live-stock feed. That is to say, it is always possible for our foodstuff to be directly or indirectly contaminated with these 12,13-epoxytrichothecene-producing microbes. It is therefore extremely important to develop a method to quantitatively and qualitatively estimate minute amounts of these toxic metabolites.

Table2. The trimethylsilylation and gas chromatographic conditions

Trimethylsilylation		Gas chromatography	
BSA	10 μl	Model	Shimadzu GC-4APF (hydrogen flame ionization)
TMCS	5 μl	Column	OV-17 1.5% (support, shimalite W)
Benzene	85 μl	Carrier gas	N_2
60°C, 15 min			

Gas chromatography and g.c.–m.s. were found to be the most reliable methods for chemically separating the 12,13-epoxytrichothecenes from each other. The gas chromatographic conditions are given in *Table 2*. The 12,13-epoxytrichothecenes which contain hydroxy groups should be protected in advance. Such samples were trimethylsilylated in benzene using *N*,*N*-bis-(trimethylsilyl)acetamide and trimethylsilyl choride by heating for 15 min to 60°C in a shield glass tube. N.m.r. analysis showed that, for example, the material obtained from nivalenol with this procedure was 3,4,15-tri(trimethylsilyl)nivalenol. 1 μl of this solution was injected into a column consisting of 1.5 per cent OV-17 with shimalite W as its support. The column temperatures used were 220 to 240°C. As shown in *Figure 1*, there was only one peak on the gas chromatogram and its retention time was 3.55 min.

The peak height corresponds to the quantity of the sample and retention time differs from sample to sample: nivalenol, fusarenon-X, diacetylnivalenol, T-2 toxin, diacetoxyscirpenol, neosolaniol, 4-deoxynivalenol and 4-deoxy-3-acetylnivalenol were trimethylsilylated under the above-mentioned conditions (trichothecin without hydroxy groups was left untreated) and their retention times were then measured by gas chromatography.

As can be seen in *Table 3*, the substances were well separated in terms of their retention times except for trimethylsilyl-T-2 and -trichothecin. A 3.50 min difference in retention time was observed for these two compounds when an SE-30 instead of an OV-17 column was used at a temperature of 100°C. Even under the previous column conditions, the two could be adequately distinguished by use of g.c.–m.s.

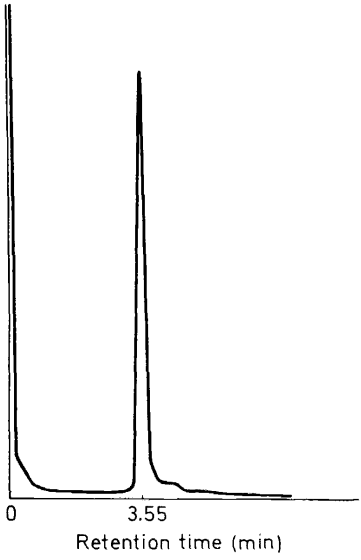

Figure 1. Gas chromatogram of nivalenol

The possibility of quantitative estimation of the 12,13-epoxytrichothecenes was now investigated. Various amounts of nivalenol, fusarenon-X and diacetylnivalenol were trimethylsilylated according to the above reported method, and 1 μl of this was injected into the column. A plot of the peak heights against the sample quantity in all cases was linear and reproducible, and could be used as the standard gauge-line for quantitative estimation. This method was then applied to estimate fusarenon-X in animal feed. The fact that the toxicity (added fusarenon-X) of the powder feed for experimental animals diminishes drastically while the feed is stored at room temperature has been known for some time. Two samples were taken: (*i*) 5 g of powdered feed (CE-2) immediately after addition of 0.2 mg of fusarenon-X, and (*ii*) the same amount of the same feed, after addition of the same amount of fusarenon-

Table 3. Retention time of 12,13-epoxytri-K. chothecenes*

Sample	Retention time (min)
Nivalenol	3.55
Fusarenon-X	3.95
Diacetylnivalenol	8.95
Trichothecin	8.55
T-2 toxin	8.55
Diacetoxyscirpenol	5.95
Neosolaniol	7.50

* Column temperature 240°C

X, left at room temperature for 2 days. Each sample was subjected to adequate extraction under agitation in a mixed solution of 10 per cent NaCl (aqueous) and 10 per cent methanol. The mixed solution was shaken well with n-hexane to remove the lipids contained in the mixed solution. Next, fusarenon-X was transferred to chloroform, and the chloroform layer was dried over anhydrous sodium sulphate and then analysed by gas chromatography.

When the extraction was performed immediately after fusarenon-X addition, 86 per cent of fusarenon-X was recovered according to a calculation based on the above-mentioned gauge-line. Setting this control level as 100 per cent, the recovery of fusarenon-X was only 75 per cent when extracted 2 days after its addition. Ueno and his colleagues[22] proved, using the rabbit reticulocyte method, that after 2 days of storing the feed lost as much as one quarter of its original toxicity. Our result, therefore, corresponded very well to the loss of toxicity estimated by the biological assay.

These results confirmed the possibility of using gas chromatography for detecting 12,13-epoxytrichothecenes in foodstuffs and cereals contaminated with sesquiterpenoid of the trichothecene group with the sensitivity of 2 to 3 μg, as long as 1 mg ml^{-1} of test material can be prepared. This level corresponds to, or even exceeds, the minimal detectable by the most sensitive biological tests with cultured cells and rabbit reticulocytes (*Table 1*). In this respect it is obvious that the gas chromatography method is much better.

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