Journal of Chromatography, 212 (1981) 370–373
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,840

Note

High-performance liquid chromatography of trichothecenes

I. Detection of T-2 toxin and HT-2 toxin

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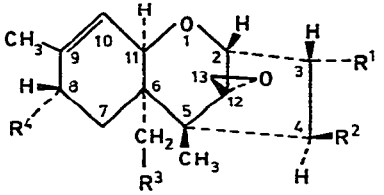
(Received March 30th, 1981)

The fungi *Fusarium tricinctum*, *F. solani* and *F. sporotrichioides* produce a large number of metabolites including the highly toxic trichothecenes diacetoxyscirpenol (DAS), T-2 toxin and HT-2 toxin[1] (Fig. 1). The absence of conjugated unsaturation in most of the trichothecenes explains their lack of UV absorption[2]. The determination of these compounds is therefore difficult. It can be achieved by thin-layer chromatography (TLC) followed by spraying with sulphuric acid[3], or derivatization with a silylating reagent and subsequent gas chromatography[4,5].



Trichothecene	R^1	R^2	R^3	R^4
T-2 toxin	OH	CH_3COO	CH_3COO	$OCOCH_2CH(CH_3)_2$
HT-2 toxin	OH	OH	CH_3COO	$OCOCH_2CH(CH_3)_2$
DAS	OH	CH_3COO	CH_3COO	H

Fig. 1. Structures of naturally occurring trichothecenes.

So far, methods for isolation of trichothecenes have relied heavily on column chromatography on alumina and silica gel, or TLC[6,7]. We have found, however, that single runs with column chromatography or TLC yield incomplete separations. Therefore multiple chromatography techniques have to be applied. Moreover, application of TLC in trichothecene isolation can be hazardous inasmuch as the operator may have direct contact with mycotoxins, especially when scratching fractions from the TLC plate.

In view of these problems with classical separation techniques, we have reconsidered the use of high-performance liquid chromatography (HPLC) for the separation and determination of trichothecenes.

EXPERIMENTAL

Production of trichothecenes by Fusaria

Flasks containing 60 g of rice and 30 ml of water were stoppered with cotton-wool plugs. After 2 h they were autoclaved for 20 min at 121°C. Then the rice was

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inoculated with a suspension of spores of *F. tricinctum* sp. 897, *F. solani* sp. 900 or *F. sporotrichioides* sp. 941 (Professor Dr. Leistner, Bundesanstalt für Fleischforschung, 8650 Kulmbach, G.F.R.) in 0.2% Tween-80 and incubated at room temperature for 6 days.

Sample preparation

The extraction of moulds has been described previously[8]. Extraction with acetonitrile–water (containing 4% of potassium chloride) was followed by partitioning against *n*-hexane and dichloromethane. After evaporation of the solvent under reduced pressure, the residue was dissolved in 400 μl methanol. A 200-μl volume of the solution thus obtained was pipetted on to a Sep-Pak C_{18} cartridge (Waters Assoc., Milford, MA, U.S.A.) and eluted with 5 ml methanol–water (80:20). The prepurified extract was transferred into a 10-ml Luer-Lok syringe containing a Swinney filter holder and a 0.5-μm Millipore filter through which the sample was filtered. After evaporation of the solvent, the residue was redissolved in a small amount of methanol and injected into the HPLC system.

For the separation of non-polar trichothecenes like T-2 toxin a modified sample preparation is advantageous. If the extract placed on the Sep-Pak C_{18} cartridge is eluted with 5 ml of 20% methanol, T-2 toxin is retained while more polar compounds pass through the bed. T-2 toxin is then eluted with 5 ml of 80% methanol. This fractionation results in a less complex chromatogram.

HPLC

Liquid chromatographic separations were performed on the apparatus recently described[9]. The prepacked μBondapak C_{18} (particle size 10 μm) column (30 cm × 7.9 mm I.D.) was obtained from Waters Assoc. The detection of the metabolites was carried out by a differential refractometer (M 401, Waters Assoc.).

RESULTS

In the course of our investigations the differential refractometer has proved to be suitable for detection of as little as 1 μg T-2 toxin per injection at the highest detector sensitivity up to semipreparative quantities of about 1 mg.

In Table I are summarized the retention times for T-2 toxin, HT-2 toxin and DAS in different methanol–water mixtures. A strong decrease in retention with increasing methanol concentration (55 to 65%) is evident. Therefore the solvent has to be prepared carefully. The retention times for the solvent containing 60% methanol are highly reproducible. Several injections of extracts from mouldy rice and pure T-2

TABLE I
RETENTION TIMES (min) OF DIACETOXYSCIRPENOL, HT-2 TOXIN AND T-2 TOXIN IN DIFFERENT METHANOL–WATER SOLVENT SYSTEMS ON A μBONDAPAK C_{18} COLUMN

Solvent (flow-rate 2.0 ml/min)	*DAS*	*HT-2 toxin*	*T-2 toxin*
Methanol–water (5.5:4.5)	12.3	19.3	30.6
Methanol–water (6:4)	9.8	13.0	17.6
Methanol–water (6.5:3.5)	8.1	10.3	13.0

TABLE II

REPRODUCIBILITY OF RETENTION TIMES FOR T-2 TOXIN, HT-2 TOXIN AND DIACETOXYSCIRPENOL IN HPLC

Parameter	*T-2 toxin*	*HT-2 toxin*	*DAS*
No. of injections, *N*	28	29	12
Retention time (min)			
Range	17.1–18.5	12.6–13.4	9.5–10.2
Mean	17.6	13.0	9.8
Standard deviation (min)	0.47	0.26	0.24
Coefficient of variation (%)	2.66	2.01	2.49

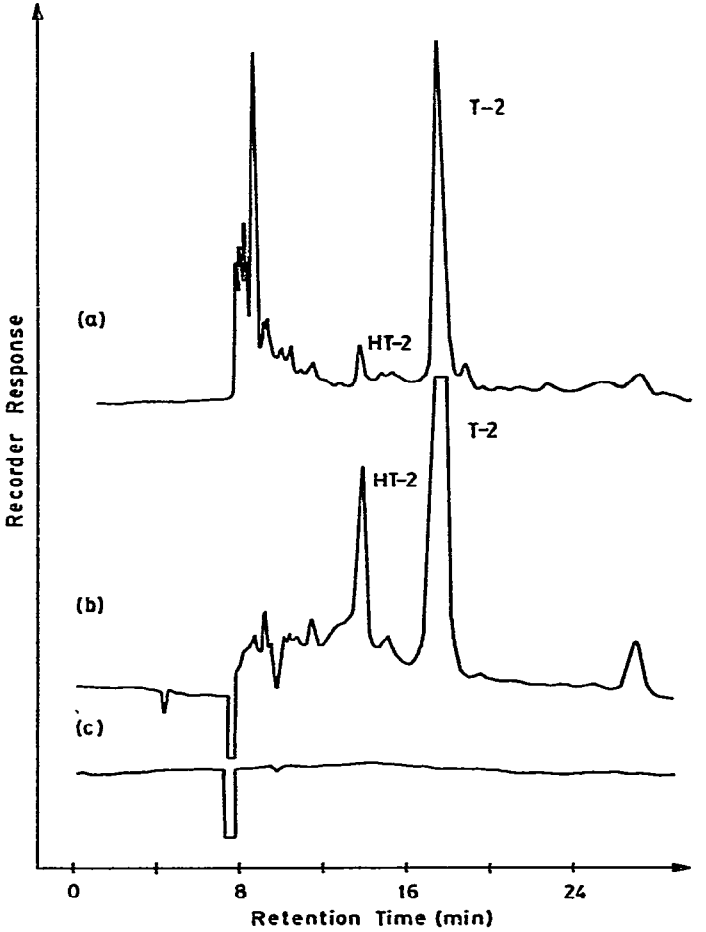

Fig. 2. Elution patterns of extracts from rice after HPLC with methanol–water (6:4) as solvent. (a) Extract from mouldy rice, using sample preparation with 80% methanol; (b) extract from mouldy rice, using modified sample preparation for removal of polar compounds with 20% methanol and elution with 80% methanol; (c) extract from non-mouldy rice.

toxin, HT-2 toxin and DAS (Makor Chemicals, Jerusalem, Israel) gave reproducible mean retention times of 17.6 min for T-2 toxin, 13.0 min for HT-2 toxin and 9.8 min for DAS (coefficients of variation 2.66, 2.01 and 2.49, respectively). The relatively high variation can be attributed to slight differences in the methanol concentration of the mobile phase (Table II).

For the separation of extracts from mouldy rice the solvent methanol–water (6:4) was suitable. The chromatograms in fig. 2 show the elution pattern for an extract from non-mouldy rice (no characteristic peaks) and for two extracts from mouldy rice which had been treated differently. The chromatograms of the extracts from cultures of *F. tricinctum* sp. 897, *F. solani* sp. 900 and *F. sporotrichioides* sp. 941 showed no remarkable differences. The accuracy of the retention times permitted a clear distinction between T-2 toxin, HT-2 toxin and DAS that was lacking in the original extracts. This result was confirmed by TLC of the fractions. At least two more metabolites (t_R 14.9 and 30.5 min), preliminarily classified as trichothecenes, have been detected on the HPLC chromatogram. Both fractions exhibit the same reaction with sulphuric acid on silica gel TLC plates (a green-yellow fluorescence) as T-2 toxin, HT-2 toxin and DAS.

ACKNOWLEDGEMENTS

This research was supported by the Bundesminister für Jugend, Familie und Gesundheit (412-6080-3/19). We thank Professor Dr. Leistner, Kulmbach, for providing us with spores of *Fusaria* and we gratefully acknowledge the microbiological work carried out by Ms. A. Bieger.

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