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Note

Determination of five trichothecenes as trimethylsilyl derivatives by gas chromatography

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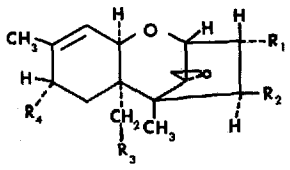
The fungi which produce toxic trichothecenes occur widely in nature both as saprophytes and plant parasites. Bilai[1] reported that *Fusarium trincinctum* has been detected in various cultured plants. This Fusarium produces a large number of metabolites including the highly toxic trichothecenes: diacetoxyscirpenol, HT_2-toxin, T_2-toxin etc.[2]. The trichothecenes consist of a family of closely related sesquiterpenoids (Fig. 1). All the naturally occurring toxins contain an olefinic bound at C 9–10 and an epoxy group at C 12–13; they may be characterised as 12–13 epoxytrichothecenes[3]. The absence of conjugated unsaturation in most of the trichothecenes explains their lack of UV absorption. The determination of these compounds is therefore difficult. Several methods have already been described for the measurement of trichothecenes including thin-layer chromatography (TLC) followed by spraying with sulphuric acid or *p*-anisaldehyde reagent[4], fluorodensitometric determination[5], high-performance liquid chromatography[6], gas chromatography with electron-capture detection[7], gas chromatography–mass spectrometry[8]. Derivatization with a silylating reagent and subsequent gas chromatography permit both flame ionization detection and quantification of most of the trichothecenes containing hydroxyl groups[9]. However, detection with gas chromatography has the disadvantage of possible misidentification because many components have identical retention times, and fatty acids in particular invariably interfere with the analysis[8].



Name	R_1	R_2	R_3	R_4
verrucarol	H	OH	OH	H
diacetoxyscirpenol	OH	OAc	OAc	H
T_2-triol	OH	OH	OH	H
HT_2-toxin	OH	OH	OAc	$OCOCH_2CH(CH_3)_2$
T_2-toxin	OH	OAc	OAc	$OCOCH_2CH(CH_3)_2$

Fig. 1. Structures of naturally occurring trichothecenes.

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In view of the problems with classical separation techniques, we have reconsidered the use of gas chromatography (GC) with flame ionization detection (FID) for the separation and determination of five trichothecenes after derivatization with bis-trimethylsilylacetamide (BSA).

EXPERIMENTAL

Standards and reagents

The trichothecenes (verrucarol, diacetoxyscirpenol, T_2-triol = scirpentriol, HT_2-toxin, T_2-toxin), the internal standard (*n*-dotriacontane), the silylating reagent (N,O-bis-trimethylsilyl acetamide) were supplied by Sigma (St. Louis, MO, U.S.A.).

Chloroform, acetonitrile, ethyl acetate, hexane (Merck, Darmstadt, F.R.G.) were distilled before use. The stock solutions of the trichothecenes were prepared in ethyl acetate (1 mg/ml) and stored at −18°C. The internal standard was prepared in *n*-hexane (1 mg/ml).

Fatty acids (lauric, myristic, palmitic, stearic, arachidic, behenic acids) were supplied by Sigma and prepared in chloroform (1 mg/ml).

Sample preparation

A 50-μl volume of each trichothecene stock solution (1 mg/ml) was introduced into a 2-ml vial. The solvent was evaporated to dryness under a gentle stream of nitrogen. To the dry sample, 50 μl of BSA were added and the capped vial heated at 100°C for 2 h. The residual BSA was evaporated under a stream of nitrogen and 100 μl of internal standard solution (I.S.) were added; 2 μl of this solution containing 1 μg of trimethylsilyl (TMS) ether derivatives of each trichothecene and 2 μg of I.S. were injected on to the gas chromatograph.

In order to separate the trichothecene peaks from interfering peaks, caused by fatty acids, 50 μl of fatty acid stock solution were added to 50 μl of trichothecene stock solution, and the same procedure was performed.

Extraction procedure

A 10-ml volume of milk containing 0.2 mg/kg (ppm) of each trichothecene was pipetted on to a Sep-Pak C_{18} cartridge (Waters Assoc., Milford, MA, U.S.A.) and eluted with 5 ml of an acetonitrile–water mixture (10:90). This extract was discarded. The trichothecenes were then eluted with 5 ml of 100% acetonitrile. The extract was transferred to a vial, evaporated under nitrogen and the derivatization was performed as above. After evaporation 20 μl of internal standard solution were added to the TMS-trichothecenes; 2 μl of this solution were injected on to the gas chromatograph.

Gas chromatography

Analyses were performed using a Hewlett-Packard model 5710 A gas chromatograph equipped with a dual differential flame ionization detector and fitted with a glass column (6 ft. × 2 mm I.D.) packed with 3% OV-17 on Chromosorb W-HP (100–120 mesh). A reporting integrator model HP 3390 A for recording chromatograms and measuring peak areas was connected. The column temperature was programmed from 230°C (2 min) to 280°C (4 min) at 5°C/min. The injector temperature was 250°C, detector temperature 300°C and the carrier gas (nitrogen) flow-rate was 17 ml/min.

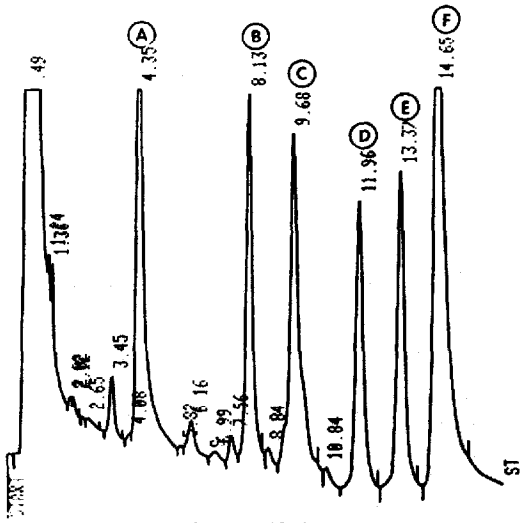

Fig. 2. Separation of the TMS derivatives (1 μg) of (A) verrucarol, (B) diacetoxyscirpenol, (C) T_2-triol, (D) HT_2-toxin, (E) T_2-toxin. The internal standard (2 μg) is *n*-dotriacontane (F).

RESULTS AND DISCUSSION

A typical chromatogram obtained from standard solution containing 1 μg of each trichothecene shows the elution pattern (Fig. 2). The accuracy of the retention times permits a clear distinction between verrucarol, diacetoxyscirpenol, T_2-triol, HT_2-toxin and T_2-toxin. Because many of the trichothecenes tested had very short retention times at 250°C, linear temperature programmation appears more suitable for their separation. In these conditions trimethylsilyl (TMS) derivatives prepared with BSA were stable and chromatographed as single peaks. The interassay reproducibility of the method with the internal standard shows that variation coefficients do not exceed 9.4% (Table I). In addition, no important interference from fatty acids has been observed. With the exception of behenic acid, these compounds did not

TABLE I

REPRODUCIBILITY OF TRICHOTHECENE MEASUREMENT

1 μg injected. Mean of 15 assays. S.D. = standard deviation.

Trichothecene	*Retention time (min) ± S.D.*	*Amount (μg) ± S.D.*	*Coefficient of variation (%)*
Verrucarol	4.35 ± 0.06	1.02 ± 0.09	9.3
Diacetoxyscirpenol	8.14 ± 0.07	1.00 ± 0.07	7.2
T_2-triol	9.69 ± 0.07	1.02 ± 0.09	8.5
HT_2-toxin	11.96 ± 0.07	1.03 ± 0.09	9.4
T_2-toxin	13.38 ± 0.08	1.01 ± 0.09	8.6
n-Dotriacontane	14.65 ± 0.05	Internal standard (2 μg)	

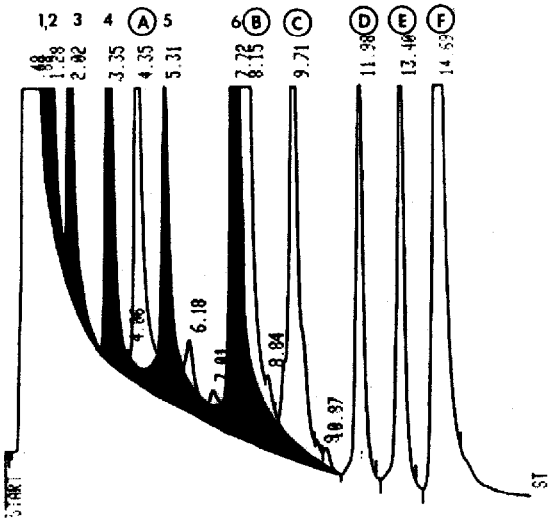

Fig. 3. Gas chromatogram of TMS-fatty acids (1 μg). 1 = lauric acid, 2 = myristic acid, 3 = palmitic acid, 4 = stearic acid, 5 = arachidic acid, 6 = behenic acid, and TMS-trichothecenes (1 μg). A = verrucarol, B = diacetoxyscirpenol, C = T_2-triol, D = HT_2-toxin, E = T_2-toxin. The internal standard is F = *n*-dotriacontane (2 μg).

exhibit, any peaks in the retention time range of the analysis (behenic acid: t_R 8.15 min; diacetoxyscirpenol: t_R 7.72 min) (Fig. 3).

This method has proved to be reliable for the detection of as little as 0.02 μg trichothecene per injection at the highest detector sensitivity. The results agree with those of Pathre *et al.*[10]. The extraction assay on Sep-Pak C_{18} with acetonitrile shows an overall recovery of *ca.* 54% for each trichothecene (Fig. 4). This relatively poor recovery at low contamination levels (0.2 ppm) could be primarily due to losses in

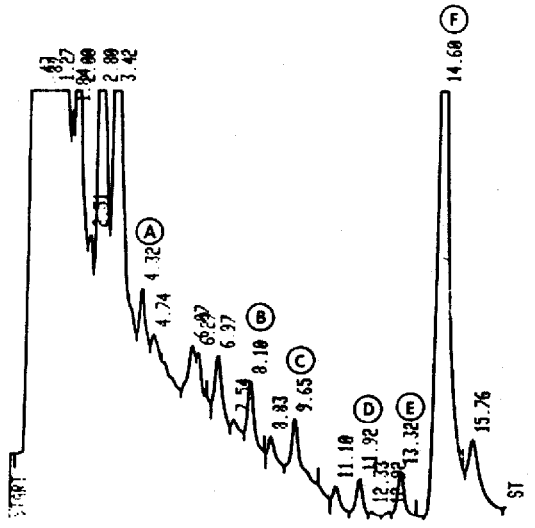

Fig. 4. Extraction assay of milk containing 0.2 mg/kg of each trichothecene captions. For peak identification, see Fig. 2.

the extraction procedure. A modification of the solvent or its proportions, or the introduction of a clean-up step, might increase the recovery percentage. However, this extraction procedure eliminates possible interference from the lipids contained in milk.

In conclusion, the gas chromatography method described is simple and sensitive. The time of analysis needed for a batch of five trichothecenes is *ca.* 17 min. The sensitivity and the accuracy of this method permits detection of about 0.1–1 ppm of each trichothecene in food. Reports in the literature on the incidence of trichothecenes demonstrate their presence in feeds and foodstuffs at levels from 50 to 1000 ppm[11]. In addition, because no acute toxicity has been demonstrated in animals fed by T_2-toxin (one of the most toxic trichothecenes) at a concentration of 15 ppm for 8 months[12], this method appears to be suitable for routine analysis of feeds suspected to be toxic.

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