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DEVELOPMENT OF A METHOD FOR THE ANALYSIS OF T-2 TOXIN IN MAIZE BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

A ground sample is homogenised with ethyl acetate, the mixture filtered, and the extract recovered by evaporation of the solvent. The residue is defatted by partition between aqueous methanol and hexane. The aqueous phase is evaporated to dryness and the residue dissolved in dichloromethane and passed through a cartridge of silica. T-2 toxin is eluted with methanol–dichloromethane, the solvents removed and the residue silylated. O-Trimethylsilyl T-2 toxin is detected by gas chromatography–mass spectrometry with monitoring of the ions at m/z 350 and m/z 436. The quantitative detection limit is 5 ppb (μg/kg) with an average recovery of 80%.

INTRODUCTION

The trichothecenes are a family of about 40 naturally occurring sesquiterpenoids produced by various species of fungi, from the genera *Fusarium*, *Myrothecium*, *Trichoderma*, *Cephalosporium*, *Verticimonosporium*, and *Stachybotrys*. These fungi attack many agricultural and plant products. However, serious contamination of feedstuffs is probably caused primarily by species of the genera *Fusarium* and *Stachybotrys*[1]. The former has been extensively implicated in various human disorders as species of *Fusaria* commonly invade cereals. Species of the latter have been implicated in outbreaks of animal and human diseases, notably equine stachybotryotoxicosis[2].

Intoxications of both human and animal populations have been noted throughout the world in temperate regions subject to cool wet periods[3]. Haemorrhagic disease, mouldy cereal amesis, akakabibyo toxicosis, alimentary toxic aleukia, stachybotryotoxicosis, and fusariotoxicosis are a few of the diseases that can be considered primarily due to trichothecene poisoning[2]. All of these occur in populations that have consumed mouldy food, usually after exposure of the foodstuffs to prolonged cool and wet weather in the field. These conditions are ideal both for growth of the fungi responsible for the diseases and also for production by the fungi of the causative agents. Many of the symptoms can be reproduced when pure trichothecenes are fed to animals, *i.e.* dermatitis, vomiting, diarrhoea, necrosis, haemorrhage, leukopenia, and shock.

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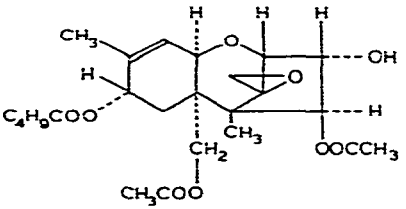

Fig. 1. Structure of T-2 toxin.

Trichothecenes are usually extracted from samples with ethyl acetate or chloroform, but more polar solvents (aqueous methanol) are required for extraction of nivalenol, fusarenone-X, and deoxynivalenol[4]. The extract is usually associated with pigmented substances and lipids which interfere with the analysis. Defatting is accomplished by partitioning the extract between a polar solvent and hexane which removes fat and pigment. The trichothecenes are retained in the polar phase. Further purification can be achieved using a silica gel column[5] or thin-layer chromatography[6]. Another approach to purification involves adsorption of the toxins on Amberlite XAD-2, followed by elution and adsorption on a florisil column[7].

Chemical analysis of the trichothecene toxins is difficult because they have no fluorescent or ultraviolet absorbing properties. They can be detected after separation on thin-layer chromatograms, by spraying with sulphuric acid and charring which causes some of the toxins to fluoresce blue under ultraviolet light. Spraying the plates with *p*-anisaldehyde reagent yields characteristic colours with some of the trichothecenes[8]. The spray reagent, 4-(*p*-nitrobenzyl) pyridine has also been used for the detection and spectrodensitometric determination of tricothecenes. The colour is based upon reaction with the 12,13-epoxy group and is sensitive for T-2 toxin (Fig. 1) to 0.1 μg per spot[9].

Even with selective detection methods, thin-layer chromatograms of natural extracts are difficult to interpret. These methods are only semi-quantitative with a detection limit of several hundred parts per billion (10^9) in foods.

Gas chromatography permits detection and quantitation of most of the trichothecenes[8] except the verrucarins and roridines. Usually acetate or trimethylsilyl derivatives are prepared and analysed on short non-polar columns (such as OV-1 phase). Detection sensitivity is better with gas chromatography, than with thin-layer chromatography, but has the disadvantage of possible incorrect identification because many components have identical retention times[6]. Separation by thin-layer chromatography prior to gas chromatographic analysis partially overcomes this problem[8].

Detection of trichothecenes has been achieved using gas chromatography fitted with an electron-capture detector[7] after preparing suitable derivatives. Although a high sensitivity is sometimes achieved, it is done at the expense of selectivity, so that further sample purification is needed. This is, however, a cheaper alternative than detection by gas chromatography–mass spectrometry (GC–MS), which has the advantage of extreme selectivity as well as sensitivity.

An alternative approach to chemical analysis is bioassay. Usually skin reactions in laboratory animals are chosen[10]. This approach only demonstrates that a skin irritant is present, which is assumed to be a trichothecene.

Two recent publications review the natural occurrence of trichothecenes[11] and the existing means for their analysis[12].

EXPERIMENTAL AND RESULTS

Gas chromatography–mass spectrometry

A Hewlett-Packard 5992A system was operated at standard autotune resolution in both the "peakfinder" mode and the "multiple ion detection" mode, unless otherwise stated. The instrument was fitted with a glass column (1 m × 2 mm I.D.) packed with 3% OV-7 on Chromosorb W AW DMCS (80–100 mesh), and programmed from 160° to 270°C at 15°C/min. Injection port temperature was 250°C. Helium was the carrier gas, at a flow-rate of 25 ml/min.

Materials and reagents

All reagents were AnalaR grade, unless otherwise stated: glass distilled water; ethyl acetate; hexane (glass distilled); methanol; chloroform; dichloromethane; sodium sulphate, anhydrous (S.L.R.); tetrachloroethylene (special for spectroscopy). N-Methyl-N-trimethylsilyl trifluoroacetamide (MSTFA) was obtained from Pierce. T-2 toxin was obtained from Makor Chemicals, Jerusalem, Israel. Stock solution made up in chloroform to concentration of 1mg/ml. Other solutions obtained by dilution.

The following equipment was used: laboratory mixer–emulsifier (heavy duty, sealed unit) fitted with a 3/4 in. tubular head (Silverson); rotary evaporator (Büchi) fitted to a vacuum water pump and used with a water bath (20°–100°C); orbital mixer; block heater (ambient to 130°C) capable of taking 0.3 ml and 1 ml Reacti-vials (supplied by Pierce); Whatman No. 1PS phase separating paper (15 cm diameter); Sep-Pak silica cartridges (Waters Assoc.); 0.3-ml and 1-ml Reacti-vials (Pierce); Pasteur pipettes, 230 mm length; 10-μl syringe (Scientific Glass Engineering).

Extraction and separation

The sample (10 g; milled in a coffee grinder), anhydrous sodium sulphate (10 g), and ethyl acetate (40 ml) were homogenised for 1.5 min in the sealed Silverson homogeniser. The mixture was filtered through Whatman 1PS paper into a round-bottomed flask and the residue washed with more ethyl acetate (2 × 15 ml), before being discarded. The filtrate was vacuum rotary evaporated at 45°–50°C just to dryness, the residue was mixed with hexane (10 ml) and 20% water in methanol (5 ml) and the mixture shaken by hand for 1 min. The hexane layer was discarded and the aqueous–methanolic phase was twice re-extracted with hexane (5 ml); the hexane extracts were discarded. The aqueous–methanolic phase was vacuum rotary evaporated at 60°–65°C to dryness.

A Sep-Pak silica cartridge was pre-washed with methanol (5 ml) and dichloromethane (10 ml). The sample residue was transferred to the syringe above the cartridge with four sequential portions of dichloromethane (4 × 0.5 ml, total volume made up to 2 ml if necessary. The solution was pumped through the cartridge, followed by dichloromethane (2 ml and then 10 ml). Eluate collection commenced and the cartridge was stripped with dichloromethane–methanol (10 ml). The eluate was vacuum rotary evaporated to dryness at 45°–50°C and the residue transferred to a 1

ml Reacti-vial with sequential portions of methanol (3 × 0.25 ml). The samples were heated at 60°C in a block heater and the solvent removed in a stream of air.

N-Methyl-N-trimethylsilyl trifluoroacetamide (25 μl) and tetrachloroethylene (50 μl) were added to the residue and the sample heated at 60°C for 30 min to form O-trimethylsilyl T-2 toxin. A 1-μl aliquot was analysed by GC–MS.

A portion of the diluted stock solution of T-2 toxin (185 ng) was mixed with tetrachloroethylene (50 μl) and N-methyl-N-trimethylsilyl trifluoroacetamide (25 μl) and heated at 60°C for 30 min. This solution was used to determine the retention time of the derivative and to calculate recovery values. Injection of an aliquot of a more concentrated solution into the GC–MS system gave the mass spectrum of O-trimethylsilyl T-2 toxin.

The mass spectrometer was operated in the selected ion detection mode for the analysis of samples and was switched on 7 min after the commencement of the gas chromatographic run. Ions *m/z* 350.0 and 436.0 (Fig. 2) were monitored with dwell times of 200 msec. O-Trimethylsilyl T-2 toxin had a retention time of 8.5 min. The sensitivity of the instrument was adjusted so that 1 ng of derivative could be detected. The response ratio between the ions was used to confirm the identity of the derivative and quantification was performed with the most sensitive ion. Typical traces obtained from maize extracts are shown in Fig. 3.

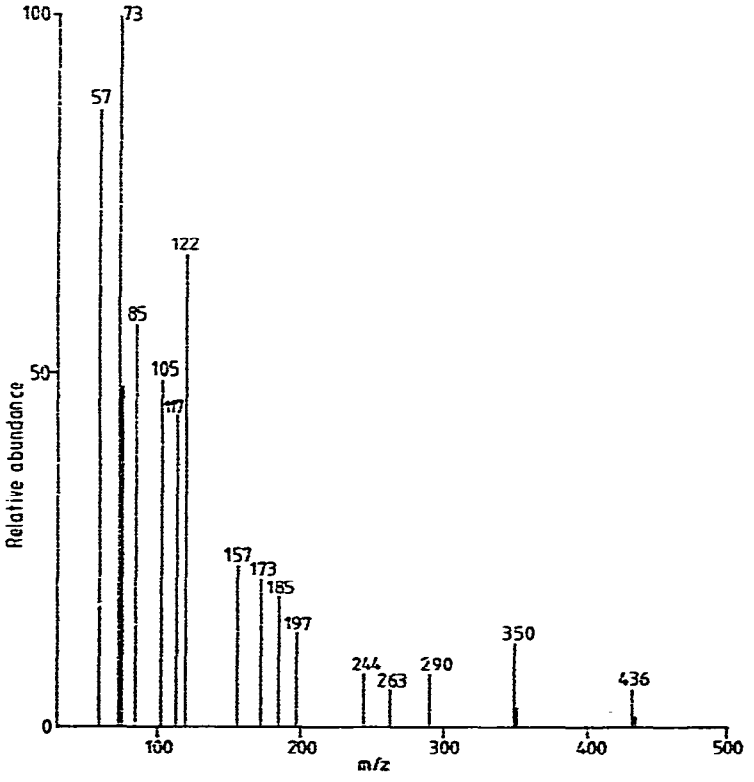

Fig. 2. Mass spectrum of O-trimethylsilyl T-2 toxin.

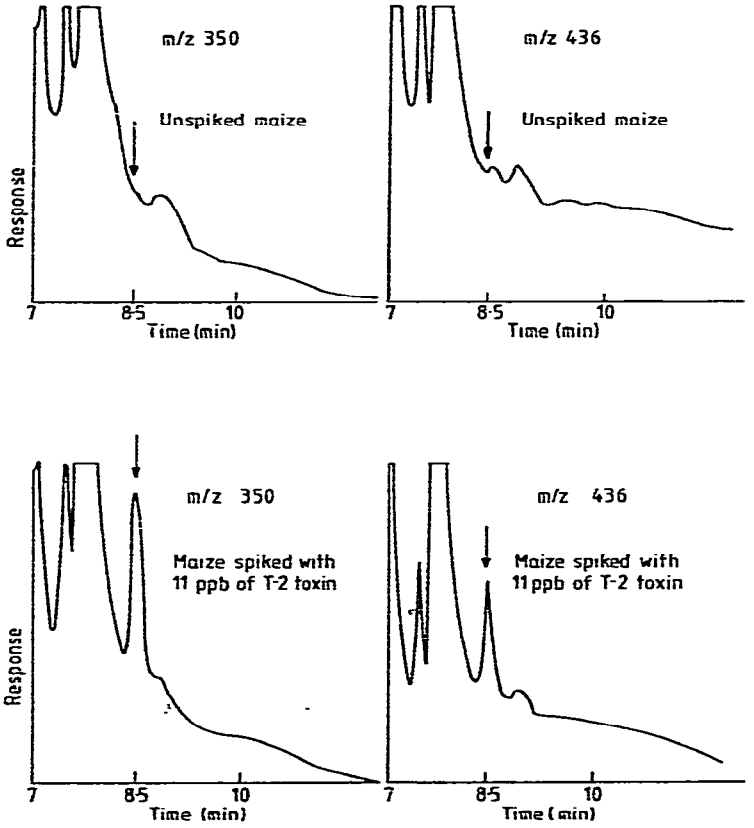

Fig. 3. Analysis of T-2 toxin as the trimethylsilyl derivative by GC–MS with selected ion monitoring. Conditions: a glass column (1 m × 2 mm I.D.) packed with 3% OV-7 in Chromosorb W AW DMCS (80–100 mesh) temperature programmed from 160°C to 270°C at 15°C/min.

Recoveries of added toxin

T-2 Toxin was added to pulverised maize at the levels shown in Table I and the samples were extracted as described. The recoveries were calculated by comparison of extracts against standards of silylated T-2 toxin, run sequentially on the gas chromatograph–mass spectrometer.

TABLE I

T-2 TOXIN IN MAIZE

Level added toxin (μg/kg)	*Recovery (%)*
368	100, 103
37	109, 97
19	70, 77
11	61, 65 60, 71

DISCUSSION

The extraction and analysis described for T-2 toxin in maize is simple and reproducible. Other workers[7] cleaned-up extracts by column or thin-layer chromatography but both of these techniques are tedious and non-reproducible.

Ethyl acetate was chosen as the extracting solvent because it was sufficiently polar to dissolve T-2 toxin but not polar enough to dissolve water-soluble materials, which could make effective separation more difficult. Most of the substituted trichothecenes, including T-2 toxin, are readily soluble in ethyl acetate or chloroform. The number of stages in the procedure was kept to a minimum to avoid undue losses of material. Separations on columns are particularly prone to cause variable recoveries when very small amounts of material are being handled. Recoveries of T-2 toxin were lower when added to samples at levels close to the detection limit than when added at the higher levels (Table I). It is likely that the defatting stage removes some T-2 toxin as it can dissolve appreciably in fat-saturated hexane, and therefore partitions between the phases rather than remaining entirely in the aqueous phase.

The extraction and separation required very selective detection to discriminate T-2 toxin from interfering material. The toxin is insufficiently volatile to pass through a gas chromatographic column but suitable derivatives like trimethylsilyl ethers or trifluoroacetyl esters may be prepared to increase its volatility. These derivatives are amenable to detection by MS which can monitor particular ions. Combined GC–MS therefore provides extremely selective detection as not only must be column retention time be correct but the ratio of responses to the monitored ions must also be correct before a peak is considered positive. The technique is also extremely sensitive as the instrument monitors only a few ions at a time. When high mass ions are examined then the chances of interference occurring are correspondingly reduced and the compound of interest can be detected in the presence of large amounts of other material. The trimethylsilyl ether of T-2 toxin was chosen as the derivative to monitor mainly because of its ease of preparation but also because of the intensity of some high mass ions in its spectrum (Fig. 2). Relatively intense ions exist at m/z 436 $(M-102)^+$, which is probably loss of the isovaleryl moiety and at m/z $(M-188)^+$. The base peak in the mass spectrum of silylated T-2 toxin is m/z 73 $(Si[CH_3]_3)^+$ which cannot be monitored as it occurs generally in any silylated compound and is therefore unselective.

Fig. 3 shows the selected ion monitor chromatograms obtained from a sample of maize and the same product spiked with T-2 toxin at a level of 11 μg/kg. The arrows on the chromatograms mark the retention time of trimethylsilyl T-2 toxin and show that the quantitation limit for this toxin is better than 5 μg/kg.

The procedure described in this report is simpler than those used by other workers[1,2] and can reach the same or lower levels of detection for T-2 toxin in maize.

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