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Note

Derivatization of the *Fusarium* mycotoxin moniliformin for gas chromatography–mass spectrometry analysis

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The *Fusarium* mycotoxin moniliformin (3-hydroxycyclobut-3-ene-1,2-dione) has been found to occur naturally in samples of fungally contaminated maize[1,2], and has been implicated as a causative agent of animal diseases[3], resulting from the feeding of moulded materials. The extraction and analysis of moniliformin is difficult and methods have relied upon high-performance liquid chromatography (HPLC) with UV detection[1] or thin-layer chromatography (TLC) with colourimetric[3–5] or fluorescence quenching[6] for visualisation. Identification has thus been based entirely on agreement of retention times (or R_F values) with authentic moniliformin and has relied on the efficacy of lengthy clean-up procedures to remove potential interferences. In this laboratory we have adopted a similar approach, and recently reported an ion-pair HPLC procedure for the analysis of moniliformin in maize[7], but this again of necessity has relied on extensive clean-up and utilised non-specific UV detection.

In order to pursue a surveillance programme to establish the incidence of occurrence of moniliformin in maize intended for human consumption (and thus to assess its significance as a mycotoxin in the food supply), it is thought essential to develop an adequate confirmatory procedure; for this purpose mass spectrometry (MS) was investigated. The relatively low molecular weight of moniliformin (MW = 98) and the absence of characteristic ions in its mass spectrum, coupled with its lack of amenability to gas chromatography (GC) indicated the need for derivatization. Trimethylsilylation, methylation (diazomethane) and trifluoroacylation were not a suitable basis for a GC–MS confirmatory procedure. However, reaction with N-methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) containing 1% *tert.*-butyldimethylchlorosilane (TBDMCS) produced a compound with a molecular weight of 453 which had a characteristic mass spectrum, was formed quantitatively and in selected ion GC–MS gave good linear calibration at low levels. This paper reports the isolation, purification by high-performance size-exclusion chromatography and structural identification by nuclear magnetic resonance (NMR) spectrometry and MS of this derivative of moniliformin, and demonstrates its potential for utilisation for a quantitative GC–MS confirmatory procedure.

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EXPERIMENTAL

Materials

Moniliformin was purchased as its sodium salt from Sigma (St. Louis, MO, U.S.A.) and the free acid was custom synthesized by Lancaster Synthesis (Morecambe, U.K.), and stored at −36°C at all times. MTBSTFA containing 1% TBDMCS was obtained from Regis Chemicals (Phase Separations, Deeside, U.K.).

Derivatization

A solution of moniliformin (free acid) in acetone (1 mg/ml) was used throughout, from which aliquots (50 μl) were withdrawn and evaporated to dryness in a vial. A volume of 50 μl of derivatizing reagent (MTBSTFA–TBDMCS) in acetonitrile (1:1) was added and the mixture heated at 100°C for 15 min in a fan-circulating oven. The derivatized moniliformin was stable for several days when stored in excess derivatizing reagent, but when purified was prone to decomposition on protracted storage in solvent.

Mass spectrometry

Electron ionizaton (EI) mass spectra were obtained on a VG 7070H mass spectrometer using direct insertion probe introduction, with a water cooled probe heated from 50°C to 250°C at 2°C/s. Chemical ionization (CI) spectra were obtained using ammonia reagent gas at an indicated source housing pressure of $5 \cdot 10^{-5}$ mbar. The mass spectrometer source was held at 200°C. Ionization was at 70eV, with a 200-μA trap current. The mass spectrometer was scanned from m/z 500 to 25 at 1 s/decade, and the spectra were acquired and processed with a VG 11/250 data system.

GC–MS was carried out with a Carlo Erba 4160 GC instrument directly coupled to the above mass spectrometer, using a 25 m × 0.22 mm I.D. fused-silica CP SIL 5CB column (Chrompack, U.K.) programmed from 150°C to 250°C at 10°C/min after an initial 2-min delay. The helium carrier gas pressure was 0.9 bar, and the injection of samples (1.7 μl) was in a split mode (20:1) with the injector temperature set at 250°C.

HPLC (size-exclusion) purification

High-performance size-exclusion LC was carried out using a Waters 6000A pump (Waters Assoc., Milford, MA, U.S.A.) and Rheodyne 7010 injection system (50 μl loop). The column was 300 × 7.7 mm PL gel 5-μm poly(styrene–divinylbenzene) of nominal pore size 50 Å obtained from Polymer Lab. (Church Stretton, Salop, U.K.). The chloroform solvent was pre-purified by passage through a column of neutral alumina to remove methanol stabiliser, and was employed at a flow-rate of 0.5 ml/min. Detection was at 260 nm with a Pye LC–UV detector. The component was collected beginning 14 min after injection thus collecting material eluted after the peak maxima to minimise trapping of the tail of the derivatizing reagent. Repetitive trapping was carried out and the isolated derivatized component was bulked, and finally re-examined by HPLC to test for the absence of derivatizing reagent.

NMR analysis

Solvent was removed from the collected HPLC fraction by small scale frac-

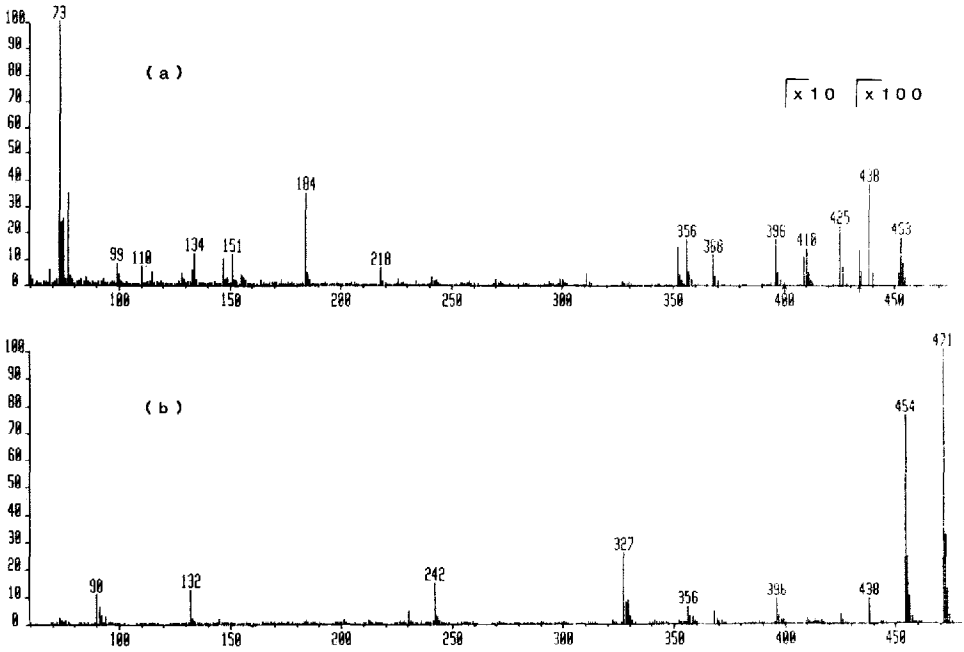


Fig. 1. Probe spectra of purified moniliformin derivative of molecular weight = 453. (a) Electron ionization. (b) Ammonia chemical ionization. Probe heated from 50°C to 250°C at 2°C/s. Mass spectrometer source at 200°C, ionization at 70 eV with scanning from m/z 500 to 25 at 1 s/decade.

tional distillation. The product was dissolved in deuterochloroform, which was also removed by distillation and finally the product re-dissolved in deuterochloroform for analysis. Proton NMR spectra were recorded at 200 MHz on a Nicolet NTC Fourier transform (FT)-NMR spectrometer, shifts being reported in parts per million relative to tetramethylsilane internal standard. Carbon-13 spectra were obtained at 50 mHz on a Nicolet NTC FT-NMR spectrometer, and at 22 MHz on a Jeol FX-90Q spectrometer.

RESULTS AND DISCUSSION

Both the electron and chemical ionization spectra of the derivatized moniliformin are shown in Fig. 1. The spectra were obtained by fractionation of the product from excess derivatizing reagent during thermal elution from the probe. Identical spectra to those produced by probe introduction were obtained by capillary GC–MS for the peak which eluted at 8 min under the conditions given in the Experimental section, and which proved to be the quantitatively formed product of the derivatization reaction.

To allow studies by NMR the derivatized product was separated from excess reagent by high-performance size-exclusion chromatography (HPSEC). Fig. 2a shows the HPSEC chromatogram of the derivatized reaction mixture. The derivatized moniliformin peak eluted on the tail of the derivatizing reagent. The component was

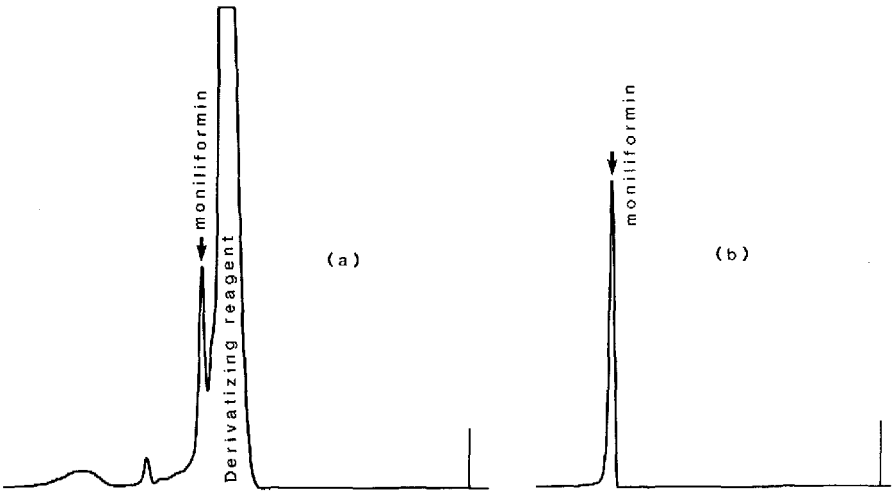


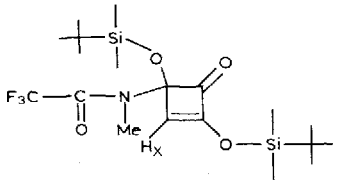
Fig. 2. High-performance size-exclusion chromatographic purification of derivatized moniliformin. (a) Derivatized reaction mixture. (b) Isolated moniliformin derivative re-chromatographed. Column: 300 × 7.7 mm PL gel 5 μm poly(styrene–divinylbenzene) of 50 Å pore size operated with chloroform solvent at a flow-rate of 0.5 ml/min. UV detection at 260 nm.

trapped slightly after elution of the peak maxima and on re-chromatographing (as shown in Fig. 2b) the isolated derivatized component was found to be free of excess reagent. Re-analysis by probe MS confirmed that the derivatized component had been isolated with high purity and that no reaction or decomposition had taken place during the chromatographic stage.

The proton and carbon-13 NMR results for the derivatized moniliformin are shown in Table I. The signals can be assigned to a structure formed from monili-

TABLE I

PROTON AND CARBON-13 ASSIGNMENTS FOR MONILIFORMIN DERIVATIVE

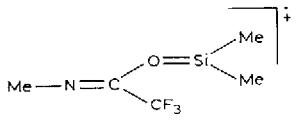


Protons	*Total number*	*Shift relative to TMS (ppm)*	*Carbons*	*Total number*	*Shift relative to TMS (ppm)*
tert.-Butyl	18	0.91	*tert.*-Butyl	8	26.3
Dimethylsilyl	12	0.10	Dimethylsilyl	3	− 2.9
			Dimethylsilyl	3	0.6
$N-CH_3$	3	3.13	$N-CH_3$	1	68.3
H_x	1	5.32			

formin by *tert.*-butyldimethylsilylation of the free hydroxyl group, and additional *tert.*-butyldimethylsilylation combined with N-methyl-trifluoroacetamide adduct formation having occurred at one of the keto groups. Such addition products from N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) reagent with carbonyl groups have recently been observed in the case of aliphatic aldehydes[8], and the proposed product from the reaction with moniliformin is of an analogous structure, and consistent with the spectroscopic evidence reported herein. In the proton NMR spectrum one major *tert.*-butyl resonance was observed at 0.91 ppm and a major dimethylsilyl at 0.10 ppm. The N–CH_3 protons are assigned to a broad signal at 3.13 ppm, the broadness resulting from hindered rotation about the N–CO amide bond which is normal for N-substituted systems. A resonance at 5.32 ppm is assigned as the olefinic proton of the silylated moniliformin which is consistent with the proposed structure in both observed shift positions and in relative intensity.

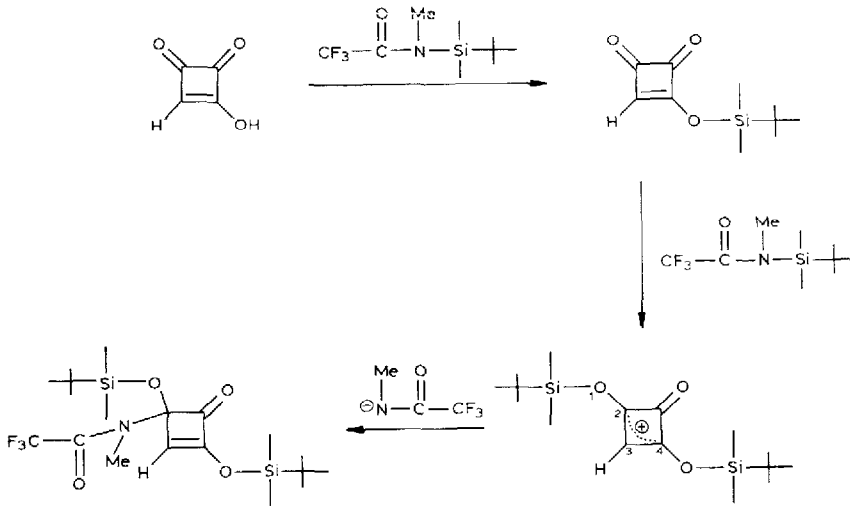
On protracted carbon-13 scanning which was necessary to accumulate sufficient NMR data for assignment on the relatively small amount of derivative available, progressive decomposition was found to occur during analysis in deuterochloroform solution. Although the products of this decomposition led to some signals being obscured, nevertheless the carbons from both the *tert.*-butyl groups produced a signal at 26.3 ppm. Two dimethyl resonances were observed at −2.9 and 0.6 ppm, the separation reflecting the close proximity of the N-methylacetamide to one of these, whilst the carbon of the N–CH_3 produced a weak signal at 68.3 ppm. The EI mass spectrum shows a weak ion at *m/z* 453, assigned as the molecular ion which is supported by the presence of fragment ions at M − 15 and M − 57. The molecular weight of 453 is confirmed in CI by the presence of an intense M + 1 ion at *m/z* 454, together with a M + 18 base peak at *m/z* 471.

The intense fragment ion at *m/z* 184 in the EI spectrum is assigned to:



formed by elimination and rearrangement of geminally placed *tert.*-butyldimethyl and N-methyl-N-trifluoroacetamide groups and subsequent elimination of a *tert.*-butyl group from the derived product. This fragment ion was observed in the MSTFA adduct products of aliphatic aldehydes[8], and is direct evidence in this case of the presence of a similar adduct. Ions at *m/z* 73, 110 and 134 are typically those seen in TMS and MTBSTFA derivatized products.

The mechanism of formation of the proposed derivative is envisaged as taking place through initial *tert.*-butyldimethylsilylation of the alcohol to form a monoderivatized product, followed by attack on one of the two carbonyls resulting in an allyl stabilised cation:



This is followed by N-methyl-N-trifluoroacetamide nitrogen anion trapping, that could occur at either the site of carbonyl derivatization (1,2-trapping) or at the delocalised allyl position (1,4-trapping). The proposed product, as opposed to other isomeric arrangements of these groups within the moniliformin molecule is sterically favoured, with the bulky *tert.*-butyl groupings being on opposite rather than adjacent carbons. Mass spectral evidence also supports geminally placed silyloxy-acetamide

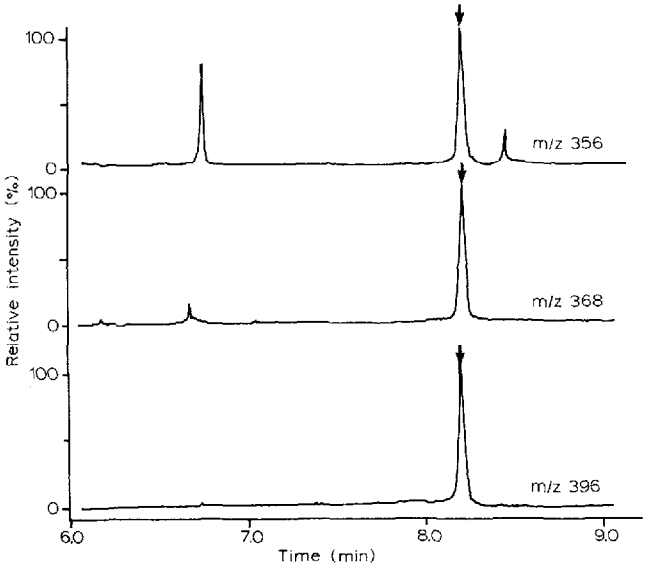


Fig. 3. Selected-ion monitoring chromatograms for derivatized moniliformin. GC–MS conditions: 25 m × 0.22 mm I.D. fused-silica CP SIL 5CB column, temperature programmed from 150°C to 250°C at 10°C/min after an initial 2-min delay (split injection). Selected-ion monitoring of *m/z* 396, 368 and 356 with an 80-ms dwell time per ion and a 20-s interscan delay.

groups, but unfortunately none of the spectroscopic evidence is capable of unequivocally establishing the substitution pattern. The derivative characterised in this paper could clearly be employed for confirmation of the presence of moniliformin in extracts from biological materials by obtaining full scan GC–MS spectra. We were however additionally interested in the possibilities of both improved sensitivity achievable through use of selected-ion monitoring, and in the possibility of direct MS quantification. Although the yield of the derivatization reaction was not determined, probe analysis did not show any evidence of underivatized moniliformin, no additional products were detected by GC, and it was thus presumed that the reaction was quantitative. In Fig. 3 selected-ion monitoring chromatograms are shown for the analysis of derivatized moniliformin standard; the monjtoring of three ions enabling a check on the specificity of analysis. Quantification was carried out by monitoring m/z 396, on the basis of peak heights. Good linear calibration was demonstrated with a regression coefficient of 0.998 (standard error = 3.74) over the range 5–500 picograms of derivatized moniliformin injected. At the lower limit this would allow sub-μg/kg sensitivity on typical gram equivalent amounts produced in concentrated and cleaned-up extracts, obtained from contaminated cereals.

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