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Note

Optimisation of conditions for the trimethylsilylation of trichothecene mycotoxins

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The trichothecenes are a group of secondary fungal metabolites with the common structural features of a tricyclic ring system containing a 9,10-olefinic double bond and a 12,13-epoxy group[1,2]. Although differing significantly in the pattern and type of other substituents, trichothecenes frequently contain one or more free hydroxyl groups. These biologically active compounds have received prominence as natural contaminants of both animal feedstuffs[3,4] and human foods[5,6], and there has been a recent plethora of publications particularly concerning the occurrence of 4-deoxynivalenol (trivially known as vomitoxin) in cereasls[7–10].

For screening of trichothecenes at trace levels in biological extracts, gas chromatography (GC) has frequently been employed, normally derivatising free hydroxyl groups as trimethylsilyl (TMS) ethers[7–9] or heptafluorobutyryl (HFB) esters[5] with detection by flame ionisation[8,11] or electron capture[5]. It was reported[12] as early as 1974 that silylation of Group B trichothecenes (those containing a hydroxyl group adjacent to the -enone function, *e.g.* 4-deoxynivalenol, nivalenol and fusarenon-X) with bis-trimethylsilylacetamide (BSA) or hexamethyldisilazane (HMDS) was incomplete but a number of subsequent papers have still appeared using inadequate derivatisation conditions[13,14]. The use of trimethylsilylimidazole (TMSIM) has been shown to be essential for this group of compounds, and although reagents containing TMSIM have been used, in general an empirical approach to derivatisation has predominated leading to a diversity of reagents and conditions appearing in the literature[11,15]. In this paper the optimum conditions are established for trimethylsilylation and additionally attention is drawn to the dangers, particularly for quantitative work, of incomplete reaction which may not always be apparent when using non-specific detection methods.

EXPERIMENTAL

Materials

4-Deoxynivalenol was obtained from Myco-Lab (Chesterfield, MO, U.S.A.), nivalenol and fusarenon-X from Wako Pure Chemical Industry (Tokyo, Japan), and derivatising reagents bis-trimethylsilyltrifluoroacetamide (BSTFA), trimethylchlorosilane (TMCS), TMSIM and a standard mixture of these reagents (Regisil 323)

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from Regis Chemicals (Phase Separations, Deeside, U.K.). Reagents were purchased in sealed ampoules which were opened immediately before use.

Derivatisation

Stock solution of the trichothecenes were prepared at concentrations of 0.2 mg/ml dissolved in chloroform–methanol (90:10) containing 0.1 mg/ml methyl stearate as an internal standard. Aliquots (100 μl) of the stock solutions contained in small vials were evaporated to dryness and treated with derivatising reagent (50 μl) and then subjected to the appropriate heat treatment.

Analysis

Capillary column GC analysis was performed using a Carlo Erba 4160 chromatograph equipped with a flame ionisation detector. A 10 m × 0.33 mm I.D. fused-silica column coated with 0.2-μm CPSIL5 (Chrompack, The Netherlands) was employed with hydrogen carrier gas at 0.3 bar pressure (approximately 1.8 ml/min.). The oven was held isothermally at 180°C and injections (1.7 μl) were in the split mode (20:1) with the injection splitter held at 250°C. Quantification was on the basis of peak heights relative to that of the internal standard.

Confirmation of identity of derivatised components was by electron impact GC–mass spectrometry (MS) using a Carlo Erba 4160 coupled to a VG 12000 quadrupole mass spectrometer. The column and GC condition were identical to those above, with the GC column directly coupled to the mass spectrometer through an all-glass transfer line heated to 250°C. Ionisation was by 70 eV electron impact at a source temperature of 200°C and with a trap current of 200 μA. The mass spectrometer was repetitively scanned from m/z 30–600 in 1 sec and the spectra were acquired and processed with a VG DS2000 data system.

RESULTS AND DISCUSSION

In preliminary experiments it was established that quantitative conversion of deoxynivalenol to its tri-TMS derivative could be achieved instantaneously with Regisil 323 at room temperature. Hence on the basis of GC peak heights relative to methyl stearate as internal standard, and estimated from the above taken to represent 100% reaction, it was possible to determine the degree of derivatisation achieved under various conditions.

Fig. 1 shows a chromatogram illustrating incomplete derivatisation obtained from treatment of deoxynivalenol with BSTFA and 323 reagent where three peaks were evident as reaction products. From the mass spectra which are shown in Fig. 2, the first component was clearly the mono-TMS derivative (MW = 368), the second was the di-TMS derivative (MW = 440) and the barely resolved shoulder on the second peak was the small amount of tri-TMS derivative (MW = 512) which was formed under these conditions. It should be noted that under conditions appropriate to the analysis for TMS derivatives, the underivatised parent did not chromatograph.

The various percentage conversions to di- and tri-TMS derivatives are shown in Table I where it is quite evident that for a variety of mixtures of derivatising reagents (many of which have been employed in the literature) and under different thermal treatments, only in the presence of TMSIM could complete derivatisation

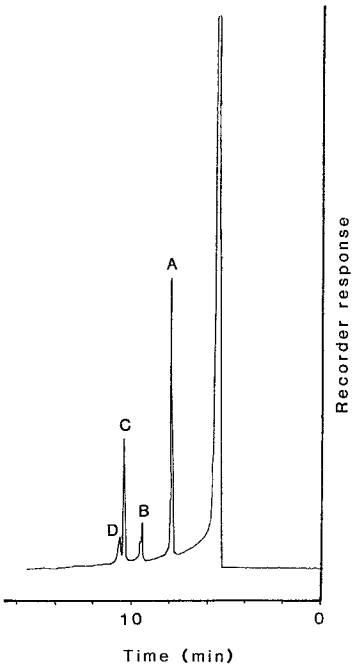

Fig. 1. FID chromatogram illustrating incomplete TMS-derivatisation. Peak identification: A = methylstearate (internal standard); B = mono-TMS-deoxynivalenol; C = di-TMS-deoxynivalenol, D = tri-TMS-deoxynivalenol. GC conditions: 10 m fused silica capillary column CPSIL5B operated isothermally at 180°C with split injection (20:1). Derivatisation with BSTFA: 323 (24:1) for 30 min at room temperature.

be achieved. With BSTFA alone despite prolonged heat treatment and in the presence both of acidic catalysts, *e.g.* TMCS and basic solvents, *e.g.* pyridine, it was not possible to achieve complete derivatisation. Monitoring by flame ionisation detection (FID) alone it might erroneously be assumed that the reaction had gone to completion, and for quantitative work errors would arise through measurement of an irreproducibly formed product.

In type B trichothecenes it is the 7-hydroxyl group which is particularly difficult to derivatise possibly because of internal stabilisation through hydrogen bonding to the adjacent 8-keto function. The TMSIM reagent recognised as being the most powerful in terms of silyl donor ability was alone amongst the reagents tested in being able to completely derivatives this hydroxyl group and was clearly the active component in the Regisil 323 mixture.

Although successful derivatisation was easily achievable with TMSIM it is not a convenient reagent as it is difficult to evaporatively remove excess. For capillary column GC analysis direct injection of TMSIM was found to damage the stationary phase leading to degraded column performance. Attempts to alleviate this problem by dilution with additional BSTFA prior to use led to successful derivatisation at a 2:1 ratio, which was insufficient to prevent column damage but increasing dilution led to a decrease in conversion to the tri-TMS derivative (Table I) and it was not possible (as would be desirable) to achieve complete derivatisation with only catalytic amounts of TMSIM.

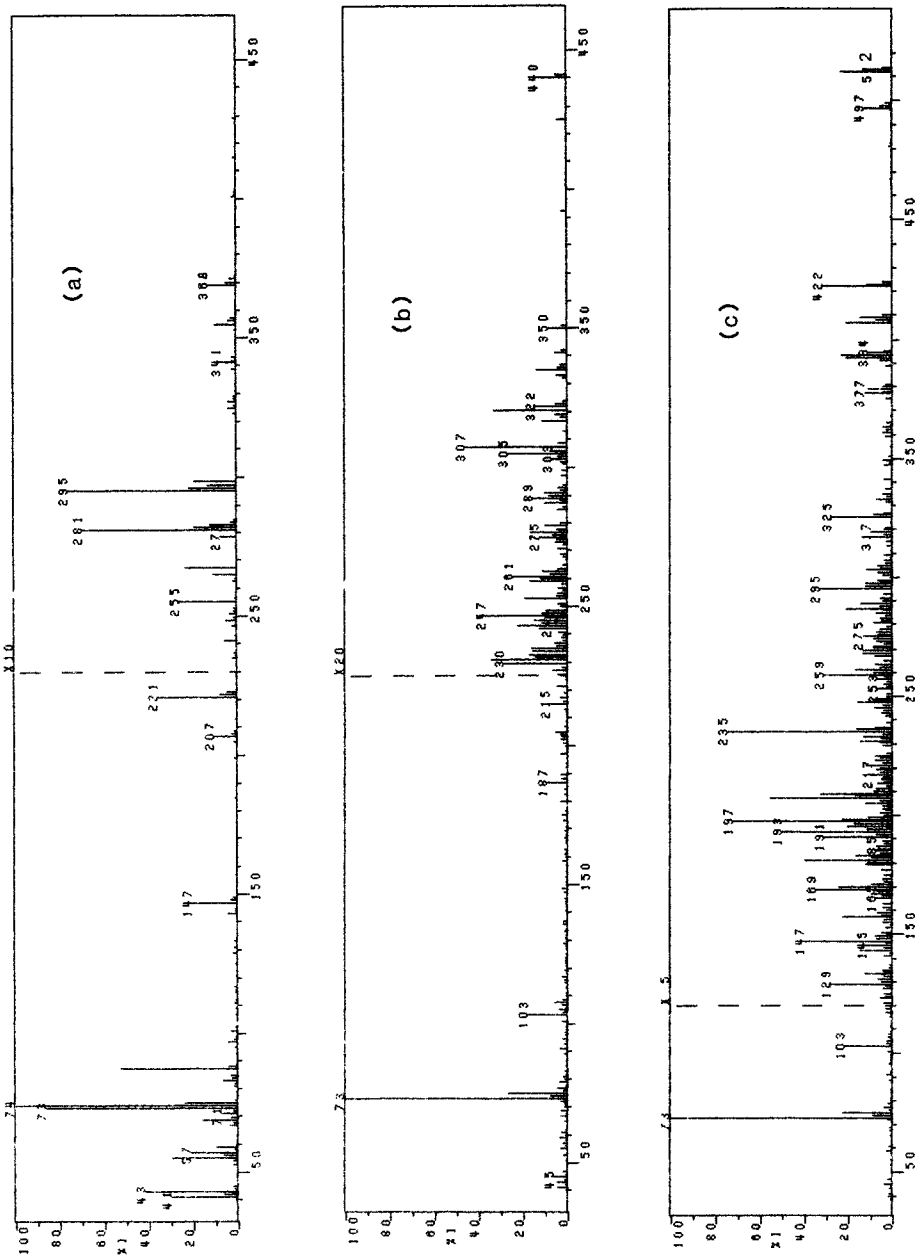

Fig. 2. Electron impact mass spectra of TMS derivatives of deoxynivalenol (a) mono-TMS derivative (MW = 368); (b) di-TMS derivative (MW = 440), (c) tri-TMS derivative (MW = 512). Spectra obtained by GC–MS under chromatographic conditions described in Experimental.

TABLE I

PERCENTAGE* DERIVATISATION OF DEOXYNIVALENOL UNDER VARIOUS REACTION CONDITIONS

Reagent	*Room temperature (30 min)*		*60°C (30 min)*		*100°C (30 min)*	
	*% Di-TMS***	*% Tri-TMS*	*% Di-TMS***	*% Tri-TMS*	*% Di-TMS***	*% Tri-TMS*
BSTFA	2	0	39	0	39	0
BSTFA–TMCS (3:2)	30	0	29	0	0.5	0***
BSTFA–TMSIM (1:1)	0	78	0	85	0	98
BSTFA–323 (1:1)	0	100	0	100	0	100
BSTFA–323 (4:1)	0	66	0	86	0	86
TMSIM	0	100	0	100	0	100

* Percentage conversion relative to methyl stearate internal standard (assuming 100% conversion to tris-TMS derivative by 323 reagent at room temperature).

** Calculated assuming relative response identical for di- and tri-derivative.

*** Decomposition product (*ca.* 17%) of close retention time to tris-TMS derivative formed.

It has previously been established that a re-arrangement of deoxynivalenol can occur under thermal treatment with a reversal in positions of the 7-hydroxyl and 8-keto groups leading to iso-deoxynivalenol[16]. Identical re-arrangement with 10–15% conversion occurred when deoxynivalenol was heated with silyating reagents with protracted heating leading to further decomposition products. This again represents a potential source of error for quantitative work, and argues strongly for derivatisation to be carried out at room temperature with TMSIM or mixtures containing TMSIM in sufficient amounts.

Using Regisil 323 at room temperature two other trichothecenes with similar functionality in the A-ring, *viz.* nivalenol and fusarenon-X both formed their respective tetra- and tri-TMS derivatives with quantitative conversion. In both cases the products were characterised by GC–MS and in no instances was there any evidence of partial derivatisation.

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