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TRIMETHYLSILYLATION AND TRIFLUOROACETYLATION OF A NUMBER OF TRICHOTHECENES FOLLOWED BY GAS CHROMATOGRAPHIC ANALYSIS ON FUSED-SILICA CAPILLARY COLUMNS

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SUMMARY

The capabilities of a number of trimethylsilylating and trifluoroacetylating agents in gas chromatographic analysis of mixtures of type A and of type B trichothecenes have been compared. Problems encountered in the analysis are mainly concerned with the decomposition of the derivatives due to imperfect removal of the excess of reagents. The application of the volatile reagent, trifluoroacetic anhydride, in combination with the acid acceptor, sodium bicarbonate, was found to be the best choice.

INTRODUCTION

Among the mycotoxins, trichothecenes form a structurally related group of toxic metabolites produced by various species of fungi found in soil, grains and foods. Their occurrence has repeatedly been reported, starting with the isolation of T-2 toxin from corn causing the death of cows in Wisconsin[1,2]. Since then, detailed studies of toxicosis have led to the identification of a large series of trichothecenes[3]. Trichothecenes have also received much attention as potential chemical warfare agents, possibly used in South-East Asia[4,5].

A number of methods for analysing these compounds have been published, of which gas chromatography in combination with mass spectrometry is the most sensitive and versatile[6]. The majority of these methods is based on the analysis of mixtures composed of only a few trichothecenes. The aim of the investigation described in this paper was to identify trichothecenes in different matrices against a background of naturally occurring quantities of these compounds. Therefore, there was an interest in the development of a universal derivatisation procedure for the quantitative analysis of samples containing both type A and type B trichothecenes (Table I). Kamimura and co-workers described such a procedure[7,8]. When applying a method comparable with this, many analytical problems were encountered, mainly concerning the derivatisation in combination with gas chromatography on fused-silica capillary columns. As the derivatisation step seemed to be of primary importance in the analytical procedure, it was decided to investigate this step more thoroughly. Zearalenone, a

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TABLE I

NAMES AND STRUCTURES OF SOME TRICHOTHECENES AND ZEARALENONE

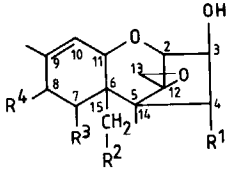
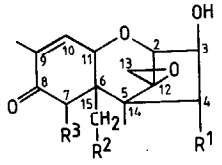
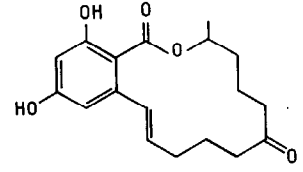
Structure	*Compound*	*Abbr.*	*R1*	*R2*	*R3*	*R4*
	*Type A*					
	T-2 toxin		$OCOCH_3$	$OCOCH_3$	H	O-isovaleryl
	HT-2 toxin		OH	$OCOCH_3$	H	O-isovaleryl
	T-2 tetraol		OH	OH	H	OH
	Neosolaniol	(NEO)	$OCOCH_3$	$OCOCH_3$	H	OH
	Diacetoxyscirpenol	(DAS)	$OCOCH_3$	$OCOCH_3$	H	H
	*Type B*					
	Nivalenol	(NIV)	OH	OH	OH	
	Deoxynivalenol	(DON)	H	OH	OH	
	Fusarenon-X	(FUS-X)	$OCOCH_3$	OH	OH	
	Zearalenone	(ZEA)				

TABLE II

NAMES OF DERIVATISING REAGENTS

Reagent	*Abbreviation*
N,O-Bis(trimethylsilyl)acetamide	BSA
N,O-Bis(trimethylsilyl)trifluoroacetamide	BSTFA
Trimethylchlorosilane	TMCS
Hexamethyldisilazane	HMDS
N-Trimethylsilylimidazole	TMSI
BSA–TMCS (5:1) Tri-Sil BT*	BT
TMSI–BSA–TMCS (3:3:2) Tri-Sil TBT*	TBT
Trifluoroacetic anhydride	TFAA
N-Trifluoroacetylimidazole	TFAI
N-Methyl-bis(trifluoroacetamide)	MBTFA

* Trade name of Pierce.

structurally different compound produced by some fungi simultaneously with trichothecenes, is included in this study.

In order to improve the gas chromatographic properties of trichothecenes, conversion into the corresponding trimethylsilyl ethers is generally carried out. The commercial availability of silylating agents, with different and generally good capabilities, probably played an important role in this. For reasons of convenience, the different reagents used in this paper will be referred to in the abbreviated forms summarised in Table II, in accordance with the *Pierce Handbook and Catalog*.

To perform gas chromatographic analysis of trichothecenes, two so-called catalysing mixtures BT and TBT are often used. The silylation mechanism and conditions that favour the reaction are complicated. The silyl-donor ability of the reagent is described in the *Pierce Handbook* and by Poole[9]. Poole stated that BSA and TMSI are potent silylating agents because the respective leaving groups, acetamide and imidazole, stabilise the partially negative charge through resonance in the transition state of the reaction.

The difference in silyl-donor ability between BSTFA and BSA may be assumed to be small, but the advantage of BSTFA over BSA is the higher volatility of the reagent and by-products giving rise to shorter retention times and consequently minor interferences during the analysis. For this reason, the silylation experiments were based on BSTFA-containing reagents. TMCS can be considered an acid catalyst and is often used in combination with HMDS. Acetylation of trichothecenes is an interesting alternative to silylation as it offers some advantages. Generally, the acetylated derivatives are more stable to heat and show less sensitivity to moisture[9].

Additionally, they are more volatile than trimethylsilyl derivatives and therefore require lower gas chromatographic temperatures. Acid anhydrides, acetylated imidazoles and acetylated amines are frequently used to derivatise trichothecenes. The acetylating agents used in this study are summarised in Table II. When using TFAA, the reaction medium will become acidic, which may give rise to a decomposition of compounds sensitive to acid. For such compounds, TFAI and MBTFA having basic leaving groups (imidazole and acetamide, respectively) are to be preferred. Furthermore, it is important to get rid of the excess of reagents because their large chemical

reactivity may have adverse effects on the subsequent gas chromatographic analysis[10]. In such cases, volatile acid anhydrides may be suitable reagents. The less involatile but reactive acylimidazole reagent renders it necessary to remove the excess by water extraction.

EXPERIMENTAL

Materials

T-2, HT-2, T-2 tetraol, NEO, DAS, DON and ZEA were supplied by Makor Chemicals (Israel). NIV and FUS-X were a generous gift from C. J. Mirocha (University of Minnesota, St. Paul, MN, U.S.A.). In all cases, stock solutions of the trichothecenes were used at concentration levels of 2–100 μg/ml acetonitrile. Toluene, acetonitrile and sodium hydrogen carbonate (Merck, Darmstadt, F.R.G.) were of analytical grade. The silylating and acylating agents (Table II) were supplied by Pierce (U.S.A.). All gas chromatographic separations were carried out on wall-coated open tubular fused-silica columns (CP-Sil 8 CB, 50 m × 0.32 mm I.D.) (Chrompack, The Netherlands).

Derivatisation procedure

Silylation. A 0.5-ml volume of the stock solution was evaporated to dryness in a 1-ml screw-capped vial by a gentle stream of nitrogen at 50°C. The sample container was carefully closed by a PTFE-faced septum to exclude exposure to the atmosphere. Quantities of 50 μl of each of the silylating agents were added. In case of on-column injection, the solution was diluted with 300 μl of toluene after completion of the reaction.

TFAA–sodium bicarbonate trifluoroacetylation. The sample was evaporated to dryness in a 1-ml vial by a gentle stream of nitrogen at 50°C and the residue was acetylated with TFAA for 30 min at 80°C in the presence of *ca.* 10 mg of sodium bicarbonate. Subsequently, 100 μl of a hexane solution containing 3.9 μg of docosane as internal standard were added at ambient temperature, followed by evaporation of the reagent for at least 10 min under a gentle stream of nitrogen. The residue was extracted for 1 min with 300 μl of toluene and the resulting solution with 0.5 ml of water to remove the residual amount of carbonate. A 100-μl volume of the toluene layer was transferred by means of a syringe to a dry Pierce reaction-vial containing anhydrous sodium sulphate. The dried sample was injected into the gas chromatographic column.

Apparatus

A Carlo Erba 5300 Mega gas chromatograph equipped with an on-column injection system, a Pye GCV with a solid injector and a Packard Becker 428 gas chromatograph with split-mode injection (ratio 1:100) were used. All gas chromatographs were equipped with flame ionization detectors. The Carlo Erba gas chromatograph was interfaced with a DEC PC 350/VG Minichrom data system. Identification was carried out on a VG 7070F mass spectrometer combined with a Varian 1400 gas chromatograph provided with a solid injector. The temperatures of the injection port and the ion source were 300 and 200°C, respectively. A VG 2050 data system was used for data acquisition.

RESULTS AND DISCUSSION

Trimethylsilylation

DAS, T-2 and ZEA were derivatised at ambient temperature with BSTFA and BSTFA–TMCS (4:1). After a reaction period of 30 min, similar gas chromatographic responses were found. After additional reaction periods at ambient temperature (3 h) or 80°C (1 h), no further increase in the peak heights of the respective derivatives could be observed. From these facts, it may be concluded that a reaction period of 30 min at ambient temperature is sufficient for a complete derivatisation of DAS, T-2 and ZEA. The reaction of DON with BSTFA and BSTFA–TMCS (4:1) showed different patterns. At ambient temperature, two derivatives were mainly found, which were identified by gas chromatography–mass spectrometry (GC–MS) as two di-TMS–DON derivatives, probably the 3,15-di-TMS and the 3,7-di-TMS. The peak assigned to the 3,15-isomer appeared first but later on or after heating at 80°C for 1 h it disappeared and was replaced by that of the 3,7-isomer (Fig. 1A). Only a small amount of 3,7,15-tri-TMS–DON was found. On addition of HMDS to BSTFA–TMCS, the 3,15-isomer was observed as the only, relative stable compound (Fig. 1B).

Tanaka *et al.*[11] and Mirocha[12] stated that DON and similar 8-keto trichothecenes react incompletely with BSA. Tanaka used a mixture of TMSI and TMCS in

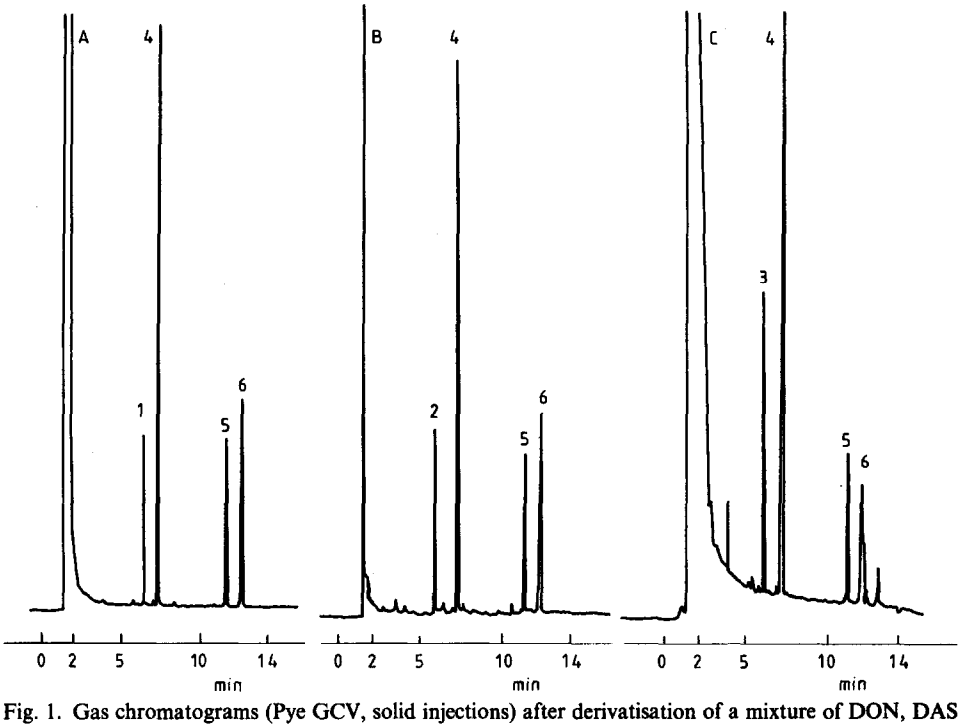


Fig. 1. Gas chromatograms (Pye GCV, solid injections) after derivatisation of a mixture of DON, DAS and ZEA with (A) BSTFA–TMCS (4:1), (B) BSTFA–HMDS–TMCS (1:1:3) and (C) TMSI–BSA–TMCS (3:3:2). 1 = Di-TMS–DON (probably 3,7); 2 = di-TMS–DON (probably 3,15); 3 = 3,7,15-tri-TMS–DON; 4 = TMS–DAS; 5 = TMS–T-2; 6 = di-TMS–ZEA.

pyridine. Mirocha prefers the TBT reagent for derivatisation of DON and NIV. Therefore, preliminary experiments with the TBT reagent were carried out. From Fig. 1C it can be seen that tri-TMS–DON, TMS–T-2 and TMS–DAS were obtained, but for ZEA the GC response was lower when compared with the results presented in Fig. 1A,B. The above differences in the derivatisation of some trichothecenes initiated a number of experiments with different silylating agents to evaluate their respective performances. These experiments were carried out on the Pye Unicam gas chromatograph using a solid injector at a temperature of 300°C. The results are presented in Table III.

It can be seen from Table III that, in the case of reagents containing TMSI in the presence of BSA or BSTFA, it seems to be essential to obtain a high response for both type A as well as type B trichothecenes. On application of the reagents without BSA or BSTFA, the responses of the type A trichothecenes decreased continuously after each injection; Table III represents only the initial experiments. Without TMSI, the reagent is unsuitable for derivatisation of the type B trichothecenes NIV and DON.

Recently, a paper was presented concerning silylation of DON with bad results on application of BSTFA[13]. It was reported that the addition of small amounts of TMSI improved the response of DON, which is in accordance with the results presented in Table III.

In the case of TMSI derivatisations, it proved to be necessary to clean the needle of the solid injector in order to obtain reproducible results with standard deviations $<15\%$. Because of highly irreproducible results, the experiments with ZEA were excluded from the table. The performance of some reagent mixtures presented in Table III were also determined on the Carlo Erba gas chromatograph using a cold on-column injection. The results obtained with BSA–TMCS, BSTFA–TMCS and TMSI–BSA–TMCS are shown in Fig. 2.

TABLE III

RELATIVE GAS CHROMATOGRAPHIC RESPONSES OF SOME TRICHOTHECENES, TOTALLY DERIVATISED BY VARIOUS TRIMETHYLSILYLATING AGENTS

Assuming that the highest peak area of the separate compounds represents 100, normalized to that of DAS; v = variable from 0 up to 30%.

Reagent mixture	*Ratio*	*Peak area of trichothecene derivative*					
		Type A				*Type B*	
		DAS	*NEO*	*HT-2*	*T-2*	*DON*	*NIV*
TMSI–BSA–TMCS (TBT)	3:3:2	100	100	86	79	91	91
TMSI–BSTFA–TMCS	3:3:2	100	96	98	87	88	85
TBT–BSTFA–TMCS	3:3:2	100	93	100	83	85	84
TMSI–TMCS–chloroform	1:0.2:9	100	80	82	58	98	100
TMSI–TMCS–pyridine	1:0.2:9	100	86	82	41	100	96
TMSI–pyridine	1:2	100	80	72	43	90	93
BSA–TMCS (BT)	5:1	100	89	92	96	v	v
BSTFA–TMCS	5:1	100	97	100	100	v	v

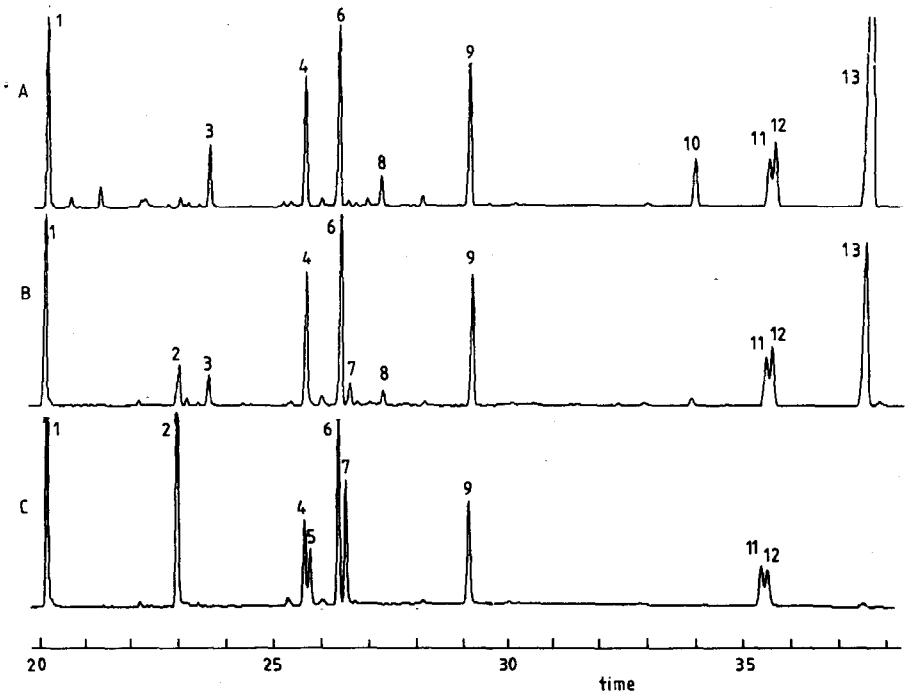


Fig. 2. Gas chromatograms (Carlo Erba, on-column injection) of a mycotoxin mixture derivatised with (A) BSTFA–TMCS (5:1), (B) BSA–TMCS (5:1) and (C) TMSI–BSA–TMCS (3:3:2). 1 = Docosane; 2 = tri-TMS–DON; 3 = di-TMS–DON; 4 = TMS–DAS; 5 = tri-TMS–FUS-X; 6 = tetra-TMS–T-2 tetraol; 7 = tetra-TMS–NIV; 8 = tri-TMS–NIV; 9 = TMS–NEO; 10 = di-TMS–ZEA (*cis*); 11 = di-TMS–HT-2; 12 = TMS–T-2; 13 = di-TMS–ZEA (*trans*).

It can be seen from Fig. 2 that the presence of TMSI in the reagent is essential for an effective derivatisation of the type B trichothecenes NIV, DON and FUS-X. However, in this case, relatively low responses were obtained for the majority of the type A trichothecenes, whereas no ZEA derivative could be observed. The derivatisation of type A trichothecenes with BSTFA–TMCS and BSA–TMCS gave identical and satisfactory results.

Whether the unsatisfactory results are due to incomplete derivatisation or to decomposition of the derivate during gas chromatographic analysis is hard to distinguish. On the three gas chromatographic systems used, the analysis of trichothecene mixtures after derivatisation with either BSA or BSTFA, in combination with TMCS, proved to be reproducible, unless an occasional injection of a mixture of trichothecenes derivatised with a TMSI-containing reagent was carried out. This resulted in a decreased gas chromatographic response of some type A trichothecenes. ZEA and partly derivatised components of DON and NIV disappeared completely. After removal of one winding of the gas chromatographic column, which probably contained residual amounts of TMSI or related degradation products, the original performances could be restored (Fig. 3). With the solid injection system, a similar effect could be obtained on application of a separate injection of large amounts of pure BSTFA as a volatising reagent.

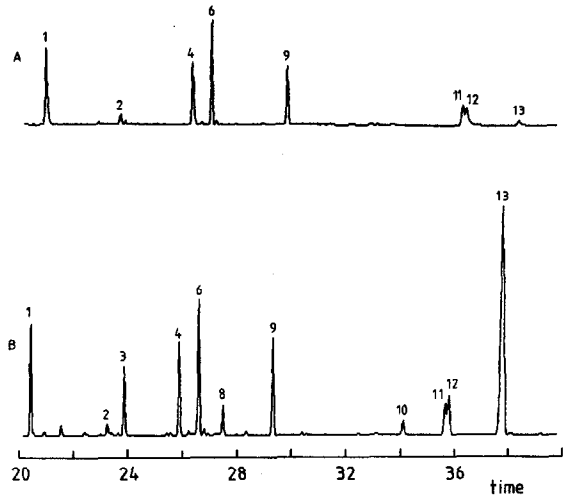


Fig. 3. Gas chromatograms of a mycotoxin mixture after BSTFA–TMCS (5:1) derivatisation. (A) The result of an injection after using the column for experiments with TMSI-containing derivatives; (B) similar to A, but after removal of the first winding of the fused-silica column. Peak numbers according to Fig. 2.

From the above-mentioned results, it can be concluded that the decomposition of derivatised trichothecenes plays an important role in the quality of the analysis. The presence of reactive residual amounts of the reagents applied will probably depend on the volatility of the reagent as well as on the injection system used, which is in turn closely related with the application of packed or capillary columns.

Trifluoroacetylation

Preliminary experiments were carried out with MBTFA to derivatise DAS. Depending on the gas chromatographic system used, three peaks representing varying amounts of three unidentified by-products in addition to that of TFA–DAS were observed in the gas chromatogram (Fig. 4). The Pye GCV gave the largest quantities of by-products. After evaporating the excess of reagent and redissolving the residue in toluene before injection into the gas chromatograph, only TFA–DAS was found.

To optimise the TFA-derivatisation of a mixture of trichothecenes, the performances of MBTFA, TFAI and TFAA were compared. In order to exclude the decomposing effects of residual amounts of reagent, these were removed before the gas chromatographic injection. MBTFA and TFAA were evaporated under a stream of nitrogen, TFAI by extraction with an aqueous 5% sodium bicarbonate solution and transferring the derivatized compounds into toluene.

For the type A trichothecenes T-2 tetraol, DAS, NEO, HT-2 and T-2, high and comparable peak areas were obtained on application of the above-mentioned three agents. The type B trichothecenes NIV, DON and FUS-X were not completely derivatised (Table IV). Some compounds gave rise to extra peaks attributable to partly derivatised trichothecenes (Fig. 5).

These results obtained on the Carlo Erba system were similar to those derived on the Pye GCV using the solid injector. As a consequence, an additional optimisation of the derivatisation technique for the type B trichothecenes proved to be

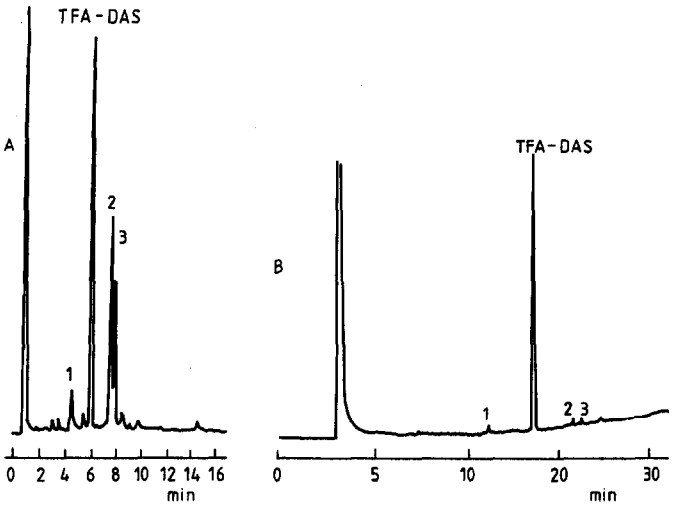


Fig. 4. Gas chromatogram of DAS derivatised with MBTFA, directly injected from the reagent mixture. Peaks 1, 2 and 3 represent unidentified by-products. (A) Pye GCV, (B) Packard Becker gas chromatograph.

necessary. Using TFAI, the water extraction step proved to be most critical, injection of some samples still leading to the destruction of column performance. After some time, the type B trichothecenes decomposed in the reaction vial, probably due to insufficient removal of the reagent or reactive by-products. TFAA was chosen for practical reasons because the excess of reagent could easily be removed completely by evaporation. During the derivatisation, pyridine and triethylamine were used as acid acceptors. As a result of this, the yield of NIV and DON derivatives increased. However, these basic solvents gave rise to broad solvent peaks. Therefore, sodium bicarbonate, being a solid acid acceptor, was preferred.

As a result, the type B trichothecenes NIV, DON and FUS-X showed much higher peak areas compared with those cases where MBTFA, TFAI and TFAA without the acid acceptor were applied (Table IV).

On application of the TFAA–sodium bicarbonate method, it proved to be necessary to liberate large quantities of enclosed TFA-trichothecenes, up to 65%.

The optimised derivatisation procedure is given in the Experimental.

As can be seen from Table IV, the TFAA–sodium bicarbonate method shows a good applicability to derivatives of both the type A and type B trichothecenes. For the type A trichothecenes, the derivatisation gave results comparable with results found in derivatisation without the addition of sodium bicarbonate; only a small loss was found, probably due to extraction with water. This method was able to derivatise ZEA, which resulted in two peaks identified by GC–MS as the *cis* and *trans* forms of di-TFA–ZEA. These derivatives proved to be very sensitive to decomposition in the case of contamination of the first winding of the fused-silica column.

In order to test the reproducibility of the TFAA–sodium bicarbonate method, peak areas obtained from a number of different standard mixtures containing type A and type B trichothecenes were compared over a period of two months. The relative standard deviation was 6–12%, as derived from thirteen experiments.

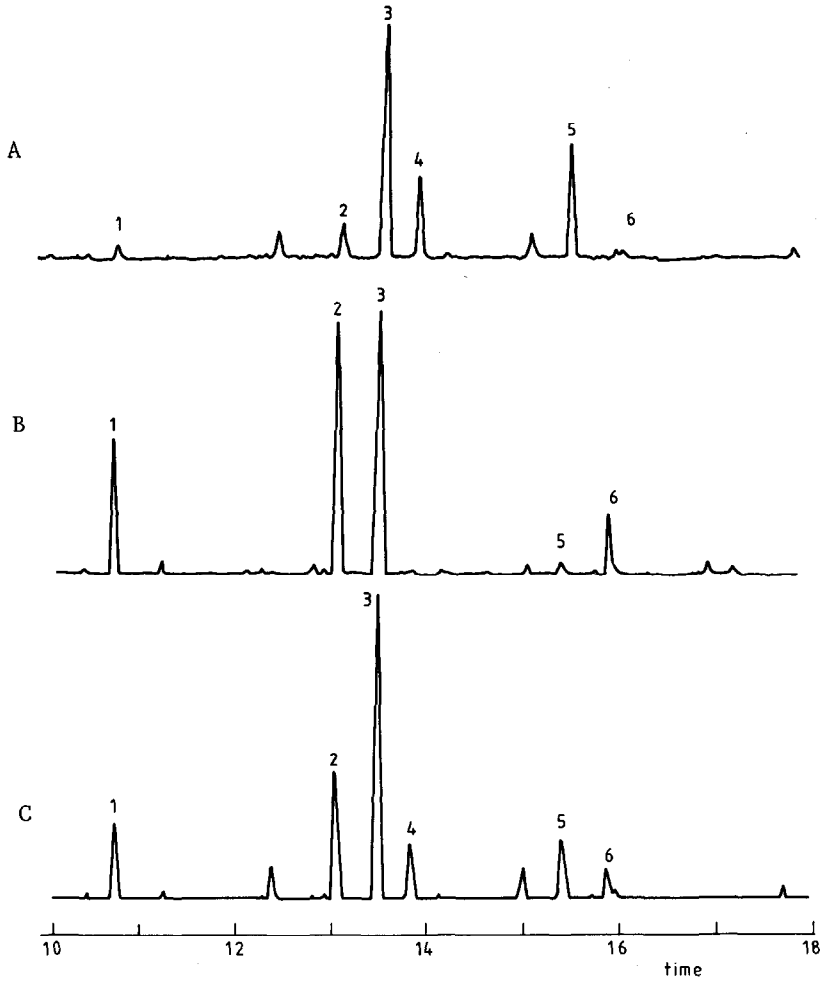


Fig. 5. Gas chromatograms (Carlo Erba) of NIV, DON, T-2 tetraol and FUS-X after derivatisation with (A) MBTFA, (B) TFAI and (C) TFAA. 1 = Tetra-TFA–NIV; 2 = tri-TFA–DON; 3 = tetra-TFA–T-2 tetraol; 4 = tri-TFA–NIV; 5 = di-TFA–DON; 6 = tri-TFA–FUS-X.

Linear calibration graphs (r = 0.999) were obtained for the corresponding trichothecenes in the range 1–25 μg, except to a minor degree for NEO (r = 0.985). This TFA-derivative is probably less stable during gas chromatographic analysis, and as a consequence provides less reproducible results. A characteristic gas chromatogram of a mixture of TFA-trichothecenes is given in Fig. 6.

CONCLUSIONS

In order to derivatise mixtures containing type A and type B trichothecenes, it is preferred to apply TFAA in combination with sodium bicarbonate. It is highly recommended to remove the excess of reagent by means of evaporation before the

TABLE IV

RELATIVE GAS CHROMATOGRAPHIC RESPONSES OF SOME TRICHOTHECENES, TOTALLY DERIVATISED BY VARIOUS TRIFLUOROACETYLATING AGENTS

Assuming that the highest peak area of the separate compounds represents 100, normalized to docosane. The gas chromatographic analyses were carried out on the Carlo Erba gas chromatograph using a cold on-column injection.

Reagent	*Trichothecene derivative*							
	Type A					*Type B*		
	T-2 Tetraol	*DAS*	*NEO*	*HT-2*	*T-2*	*DON*	*NIV*	*FUS-X*
MBTFA	99	100	95	100	100	10	5	12
TFAI	100	99	97	93	97	96	63	95
TFAA	100	94	93	96	95	32	46	45
TFAA–sodium bicarbonate	95	89	92	88	82	100	100	100

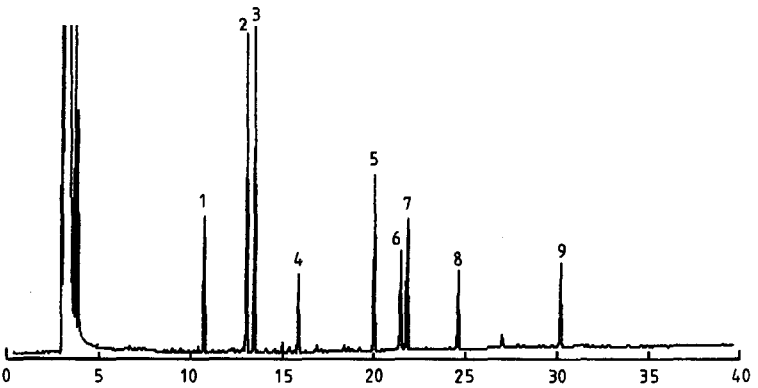


Fig. 6. Gas chromatogram (Carlo Erba) of a trichothecene mixture after derivatisation using the TFAA–sodium bicarbonate method. 1 = Tetra-TFA–NIV; 2 = tri-TFA–DON; 3 = tetra-TFA–T-2 tetraol; 4 = tri-TFA–FUS-X; 5 = docosane; 6 = di-TFA–NEO; 7 = di-TFA–DAS; 8 = TFA–HT-2; 9 = TFA–T-2.

gas chromatographic analysis is carried out. The main disadvantage of the majority of other trifluoroacetyl as well as trimethylsilyl agents to derivatise such mixtures of trichothecenes, concerns the possibility of a decomposition of the derivatised compounds mainly due to the presence of residual amounts of the reactive compounds in the injection part of the gas chromatograph. This gives an unreliable performance for multi-trichothecene analysis.

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