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DETECTION OF TRACE LEVELS OF TRICHOTHECENES IN HUMAN BLOOD USING CAPILLARY GAS CHROMATOGRAPHY–ELECTRON-CAPTURE NEGATIVE ION CHEMICAL IONISATION MASS SPECTROMETRY

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SUMMARY

A sensitive and selective method has been developed for the simultaneous detection in blood of eleven trichothecenes of widely varying polarity. The procedure involved precipitation of blood proteins with acetone followed by a clean-up using reversed-phase Sep-Pak C_{18} cartridges. The extracted trichothecenes were derivatised as their pentafluoropropionyl esters, separated using capillary gas chromatography and detected using electron-capture negative ion chemical ionisation with methane reagent gas and selected-ion monitoring. Optimum sensitivity and selectivity were obtained using low source temperatures (60°C indicated) and high source pressures (1 Torr indicated). Detection limits on 1-ml blood samples were in the range 0.1–5 ppb. The method was readily adaptable to the detection of other trichothecenes. A protocol was used which minimised the risk of cross-contamination. The method was validated in collaborative studies by the successful analysis of 42 blood samples spiked and submitted blind by two independent laboratories for analysis.

INTRODUCTION

The trichothecenes are a group of secondary fungal metabolites with the common structural feature of the tetracyclic 12,13-epoxytrichoc-9-ene nucleus shown in Fig. 1. Trichothecene mycotoxins are produced predominantly in nature by Fusarium moulds and have been implicated in outbreaks of human and animal diseases following the ingestion of mouldy food and animal feedstocks[1–3]. More recently, trichothecenes have assumed prominence following reports that the trichothecene mycotoxins T-2, HT-2, diacetoxyscirpenol (DAS), nivalenol (NIV) and deoxynivalenol (DON) were the toxic components of a new chemical warfare agent frequently described as "Yellow Rain"[4,5]. For the retrospective investigation of human exposures,

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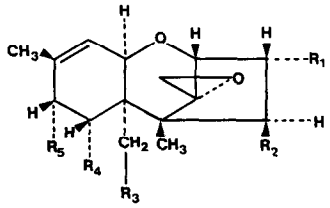

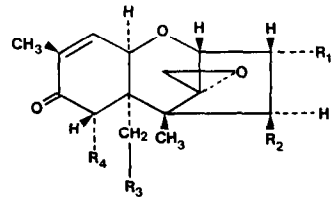

	R_1	R_2	R_3	R_4	R_5
T-2	OH	OAc	OAc	H	O $COCH_2CH(CH_3)_2$
HT-2	OH	OH	OAc	H	O $COCH_2CH(CH_3)_2$
T2-triol	OH	OH	OH	H	O $COCH_2CH(CH_3)_2$
T2-tetraol	OH	OH	OH	H	OH
DAS	OH	OAc	OAc	H	H
15-MAS	OH	OH	OAc	H	H
Scirpentriol	OH	OH	OH	H	H
VERR	H	OH	OH	H	H
7-OH DAS	OH	OAc	OAc	OH	H

	R_1	R_2	R_3	R_4
NIV	OH	OH	OH	OH
DON	OH	H	OH	OH
15-Ac DON	OH	H	OAc	OH

Fig. 1. Chemical structures of the trichothecene mycotoxins discussed in the text. 7-Hydroxy-DAS is used as an internal standard.

highly sensitive (1–10 ppb*) and selective analytical techniques were required for the simultaneous detection of a range of trichothecenes in blood.

Previous workers have developed a range of analytical methods for the assay of trace levels of trichothecenes in a variety of matrices, with much of the interest centred on recovery from grain samples. Techniques used have included thin-layer chromatography (TLC)[6], high-performance liquid chromatography (HPLC)[7–10] and gas chromatography (GC) of derivatised trichothecenes[4,5,11–28]. The derivatives used have typically been trimethylsilyl (TMS), heptafluorobutyryl (HFB) or trifluoroacetyl (TFA), while detection has been by flame ionisation (FID), electron-capture (ECD) or mass spectrometry (MS) in electron impact or positive ion chemical ionisation with selected-ion monitoring. For the present application, where high sensitivity (less than 10 ppb) and selectivity were required on small samples (1–2 ml) of high complexity (blood), it was considered that the technique should possess the highest attainable sensitivity and selectivity. Electron-capture negative ion chemical ionisation (ECNICI) MS, as reported by Hunt *et al.*[29,30], offered the potential required. This technique has been independently applied by Rothberg *et al.*[31] to the detection of trichothecenes in corn. In the present paper we describe conditions for the extraction of trichothecenes of a wide range of polarity from blood, their derivatisation, separation and detection using ECNICI-MS along with the protocol which should be adopted for unambiguous results at trace levels.

* Throughout this article the American billion (10^9) is meant.

EXPERIMENTAL

Materials

Deoxynivalenol (DON) was obtained from Myco-Lab (Chesterfield, MO, U.S.A.). 15-Acetyldeoxynivalenol was a gift from Agriculture Canada. Other trichothecenes mentioned in the text were prepared and purified at the Chemical Defence Establishment (Porton Down, U.K.). Sep-Pak C_{18} cartridges were purchased from Waters Assoc. (Stockport, U.K.). Pentafluoropropionyl imidazole (PFPI) was obtained from Pierce and Warriner, Chester, U.K. With the exception of toluene (Analar Grade, BDH, Poole, U.K.), all solvents were purchased from Fisons (Loughborough, U.K.) either as Distol reagent grade or HPLC grade in the case of water and acetonitrile.

A first set of 22 blood samples was prepared at the United States Army Research Institute for Infectious Diseases (USAMRIID), Fort Detrick, MD, U.S.A. Expired blood-bank blood was used to which sodium fluoride (0.25% w/v) had been added to inhibit esterase enzymes. A computerised random number generator was used to code the samples. The master code was known only to staff at USAMRIID. Samples were frozen after preparation, transported to the U.K. and stored at −20°C for 3–4 months prior to analysis.

A second set of twenty blood samples was prepared at the Centre for Disease Control (CDC), Atlanta, GA, U.S.A. Heparinised normal blood was used with added sodium fluoride. Samples were transported to the U.K., refrigerated but unfrozen, and were then stored at −20°C for one month until analysis.

Extraction and clean-up

Disposable materials were used where possible to minimise any possible sources of cross-contamination. New vials were used for the derivatisation of each unknown sample. Equipment which was used repeatedly, such as the glass vapour duct of the rotary evaporator and the needles of the sample concentrator, were exchanged after working up each sample and cleaned. All glassware was cleaned by soaking in sodium hypochlorite solution followed by Decon-90 laboratory detergent, then rinsed in tap water, distilled water and ethanol before heating at 200°C for several hours in a vacuum oven. Standard solutions were prepared and stored in a laboratory separate to that used for the clean-up. Before the work-up of each unknown sample, a blank sample of water and solvent was taken through the clean-up procedure as a check against false positives.

Whole blood (2 ml) was pipetted into a 15-ml centrifuge tube, acetone (4 ml) was added, and the two were mixed well using a vortex mixer. After centrifuging, the supernatant was transferred to a clean centrifuge tube and the procedure repeated on the residue using a further 4 ml of acetone. The acetone was removed under a gentle stream of nitrogen at 60°C. To the aqueous residue was added water (2 ml) and the solution loaded onto a Sep-Pak C_{18} cartridge using a syringe. The centrifuge tube was rinsed sequentially with water (2 ml), methanol–water 40:60 (2 ml) and methanol–water 90:10 (2 ml), and each passed in turn through the Sep-Pak cartridge. The water eluate was discarded. The 40:60 eluate containing T-2 tetraol, scirpentriol, NIV and DON, was collected in a 50-ml round-bottomed flask and concentrated to dryness at 30–40°C on a rotary evaporator. The 90:10 eluate was collected in a 15-ml

screw-capped tube, diluted to 50:50 with water (1.6 ml) and extracted with dichloromethane (2 × 5 ml) by tumbling for 10 min. The combined extracts were dried by passing through anhydrous sodium sulphate (2 g) contained in a disposable 5-ml plastic pipette tip, and collected in the 50-ml round-bottomed flask containing the dry residue from the 40:60 eluate. The sodium sulphate was washed with an additional 5 ml of dichloromethane and the combined extracts concentrated to dryness on a rotary evaporator. The residue was transferred to a 1-ml vial (Reactivial, Pierce and Warriner) using methanol (3 × 1 ml), the solvent being removed in a gentle stream of nitrogen at 60°C. The vials were then capped, sealed in plastic bags containing silica gel and stored at 4°C until analysis.

Derivatisation

To the dried residue from the clean-up was added acetonitrile (0.1 ml). The sample was warmed to 60°C for 2 min, followed by vigorous shaking for 1 min. Toluene (0.1 ml) was then added and the sample shaken for a further 1 min. PFPI (25 μl) was added and the sample heated at 60°C for 1 h using a Reacti-Therm heating module (Pierce, Rockford, IL, U.S.A., P/N 19790). A 1-μl aliquot was then analysed within 5 min. Both the acetonitrile and toluene solvents were spiked with the 7-hydroxy-diacetoxyscirpenol internal standard at the same 30 pg/μl level. The choice of PFPI as derivatising reagent was dictated by the upper mass limit of the mass spectrometer (*viz.* *m/z* 1000). On an instrument with an extended mass range, other workers[32] have produced comparable sensitivity using heptafluorobutyryl (HFB) derivatives, which confirmed our experience with HFB derivatives of trichothecenes containing one or two hydroxyl groups per molecule. Preliminary work with trifluoroacetyl (TFA) derivatives indicated a significant loss of sensitivity (by a factor of 3–5) compared with the pentafluoropropionyl (PFP) derivatives. Other electrophores, such as perfluorobenzoyl (PFB) and pentafluorodimethylsilyl (flophemsyl) groups, were briefly investigated for the mono- and dihydroxy species, but proved difficult to chromatograph within the column temperature limits.

Analysis

All analyses were performed using a Finnigan 4510 gas chromatograph–mass spectrometer. A 12 m × 0.2 mm I.D. fused-silica column coated with 0.33 μm cross-linked SE-54 (Ultra Series, option 105, Hewlett-Packard, Winnersh, U.K.) was used with 99.996% purity helium carrier gas (Air Products, Bracknell, U.K.) at 1.4 bar pressure, this corresponding to a column flow of *ca.* 3 ml/min at 50°C. Splitless injections of 1 μl were made using a split delay of 30 s with the injector maintained at 220°C. A septum purge of 1 ml/min was employed. Septa (Finnigan MAT, Hemel Hempstead, U.K.) were solvent-extracted with methanol prior to use and renewed daily. The oven temperature was held at 150°C for 2 min followed by temperature programming at 10°C/min to 290°C. The column was inserted directly into the mass spectrometer source and the mass spectrometer transfer line was maintained at 260°C. Because of the thermal lability of the derivatives chromatographic conditions and interface temperatures were critical in achieving the detection limits quoted later.

The mass spectrometer was operated in the negative ion mode using CP Grade methane (BOC, Wembley, U.K.) as chemical ionisation reagent gas. An ion source pressure of 0.95–1.00 Torr (indicated) was employed. The source temperature was

60°C (indicated). An electron energy of 150 eV was used. Both the carrier and reagent gases were supplied using two-stage regulators with stainless-steel diaphragms (Model 11, Scott Environmental Technology, Plumsteadville, PA, U.S.A.). The instrument was routinely mass-calibrated to *m/z* 916 in the electron impact mode using perfluoroheptyl triazine introduced through the GC inlet. Ion transmission efficiency was optimised on a daily basis using bleed and background peaks. The instrument was operated in the selected-ion monitoring (SIM) mode. The masses monitored for each of the twelve species of interest are shown in Table I; those marked with an asterisk were useful only at concentrations in excess of *ca.* 10 ppb. The total scan time for ions of interest was *ca.* 1 s. The masses monitored were split into three groups by retention time, and changed under data system control, to allow longer dwell times on the ions of interest in each portion of the chromatogram.

TABLE I

IONS USED FOR SELECTED-ION MONITORING IN NICI MODE

Ions marked with an asterisk are only useful at concentrations greater than 10 ppb.

Species	*Ions monitored (m/z)*
T-2	612*, 592, 572*, 545*, 530, 486
HT-2	716, 696, 678*
T-2 triol	820, 800
T-2 tetraol	882, 862, 719, 536*
DAS (diacetoxyscirpenol)	492, 430, 386*
15-MAS (monoacetoxyscirpenol)	616, 596
S-3OL (scirpentriol)	720, 700
NIV (nivalenol)	896, 876*
DON (deoxynivalenol)	734, 714
15-Acetyl-deoxynivalenol	630, 610*
VERR (verrucarol)	558, 538
7-Hydroxy-DAS (internal standard)	654, 592

To expedite data reduction the automatic search and quantitation software supplied (AUTOQU.PR Finnigan MAT) was used. Acceptance criteria of 4:1 signal-to-noise ratio and a 10% window on the standard retention times were employed for sample analysis. These criteria were deliberately low to eliminate the risk of false negatives being declared by the data system. Manual inspection was then confined to machine-declared positives, which were then subjected to stringent ion ratio and retention time tests (± 1%) before acceptance. Quantitation was based on a peak area basis relative to the internal standard. On each day samples were analysed, 10 ppb spikes were also run to assure system sensitivity and provide response factors for quantitation. As observed by Gilbert *et al.*, repeated injections of solutions containing excess imidazole reagent caused column degradation. The performance of the column was restored by removal of the first 30 cm of column, this being necessary at *ca.* 10-day intervals[27]. Similarly, mass spectrometer ion volumes were changed after *ca.* 50 injections.

Results validation protocol

A trace analysis protocol must include stringent precautions to minimise false results, and allow any possible contamination incidents during work-up or analysis to be recognised. The following procedure was adopted:

(1) During extraction, a high ratio of spikes and blanks to samples was incorporated. Each batch of extractions was worked-up as follows: (a) spiked sample (for sensitivity validation); (b) glassware blank; (c) sample A; (d) glassware blank; (e) sample B; (f) glassware blank, etc.

(2) On a daily basis, solutions containing only the internal standard were derivatised and run immediately before each extract (or blank) in the supplied batch. This test confirmed that the analysis equipment was free from contamination.

This protocol drastically reduced sample throughput, although it allowed high confidence to be attached to the results derived. Its use was validated by the collaborative exercises described later.

RESULTS AND DISCUSSION

Extraction and clean-up

The efficient recovery of a range of trichothecenes and their hydrolysis products is complicated by the considerable variation in physicochemical properties. Differences in polarity are reflected in their chromatographic properties, *e.g.* R_F values on silica gel, using chloroform–methanol 95:5 as eluent range from 0.56 for T-2 to 0.03 for nivalenol and T-2 tetraol. Solvents of high polarity are, therefore, required to recover the latter from normal phase silica gel on which they also show a tendency to "tail", owing to their poor solubility in most aprotic solvents. Preliminary experiments established that T-2 tetraol and nivalenol are inefficiently recovered from aqueous solutions by XAD-2 resin, neither could they be extracted from aqueous solution, *e.g.* with chloroform. They were, however, well retained by reversed-phase silica gel, and were easily recovered using methanolic eluents. The procedure adopted utilised clean-up on disposable reversed-phase Sep-Pak C_{18} cartridges. Toxins were conveniently fractionated into non-esterified trichothecenes (T-2 tetraol, scirpentriol, NIV and DON) by eluting with methanol–water 40:60, and partially esterified trichothecenes (T-2, HT-2, DAS, MAS, *etc.*) by eluting with methanol–water 90:10. The latter were then extracted into dichloromethane prior to concentration to eliminate any possibility of hydrolysis, which was sometimes observed on concentrating aqueous methanolic solutions. Overall recoveries from blood (Table II) were acceptable over the range, and were limited by the initial extraction from blood.

Derivatisation and chromatography

Upon derivatisation with PFPI, the trichothecenes showed a range of reactivity. Monohydroxylic species such as T-2 and DAS were efficiently acylated after *ca.* 20 min at 60°C, with prolonged high temperature reaction (*i.e.* 100°C) leading to degradation, whilst polyhydroxylic trichothecenes such as nivalenol required 60 min at 100°C to achieve maximum yield. Since our requirement was for a method of simultaneous screening for a range of trichothecenes a compromise of 60°C for 60 min was used. The reduced yields of the more highly acylated species was offset to a larger extent by their higher sensitivity towards detection using negative ion tech-

TABLE II

RECOVERIES OF TRICHOTHECENES FROM BLOOD AND GC–MS DETECTION LIMITS (S/N = 5:1) IN BLOOD AND SPIKED SOLVENTS

Verrucarol was added to the study at a later stage in the work and only the overall method detection limit was determined.

	Detection limits		*Recovery (%)*
	Solution (pg)	*1-ml blood (ppb)*	
T-2	10	10	20
HT-2	1	0.5	40
T-2 triol	1	0.5	40
T-2 tetraol	0.1	0.1	20
DAS	5	2	50
15-MAS	1	0.5	40
VERR	–	0.2	–
S-3OL	0.1	0.1	20
15-AcDON	1	1	20
DON	<0.1	<0.1	>20
NIV	0.1	0.5	40

niques. Although the precise percentage derivatisation was not known, it was sufficient to achieve the required sensitivity and reproducibility for the level of quantitation required.

A typical capillary chromatogram of a 10 ppb trichothecene blood spike and its associated blank is shown in Fig. 2, clearly demonstrating baseline resolution of all trichothecenes and freedom from interference in a blood matrix. The standard operating conditions were not consistent with achieving a "solvent effect" with the solvent system in use. This was reflected in peak broadening for the early eluting species. However, lowering the initial oven temperature to 80°C at which a solvent effect would be expected led to reduced sensitivity and longer analysis times with an insignificant improvement in reproducibility. This suggests that processes other than injection, such as variable extraction and derivatisation, or on-column degradation are more important in determining quantitative reliability in this case.

Mass spectrometry

The negative ion MS parameters were optimised using acetonitrile–toluene solutions spiked at the picogram level with pure trichothecenes. The effect of the ion source temperature on the negative ion spectra was dramatic. Under the usual ion source conditions where an indicated temperature of *ca.* 150°C would be employed, the spectra consisted essentially of the non-specific ion *m/z* 163 ($CF_3CF_2CO_2^-$) from the derivatising agent, indicating that dissociative electron capture had occurred. However, as the source temperature was lowered to 100°C, molecular ions were observed and at 50–60°C these were the base peak for several of the species (see Fig. 3). Fig. 4 further illustrates this behaviour for the trichothecene HT-2 at source temperatures over the range 60–100°C. These source temperatures are quoted as dial settings on the mass spectrometer and actual temperatures within the ioniser are

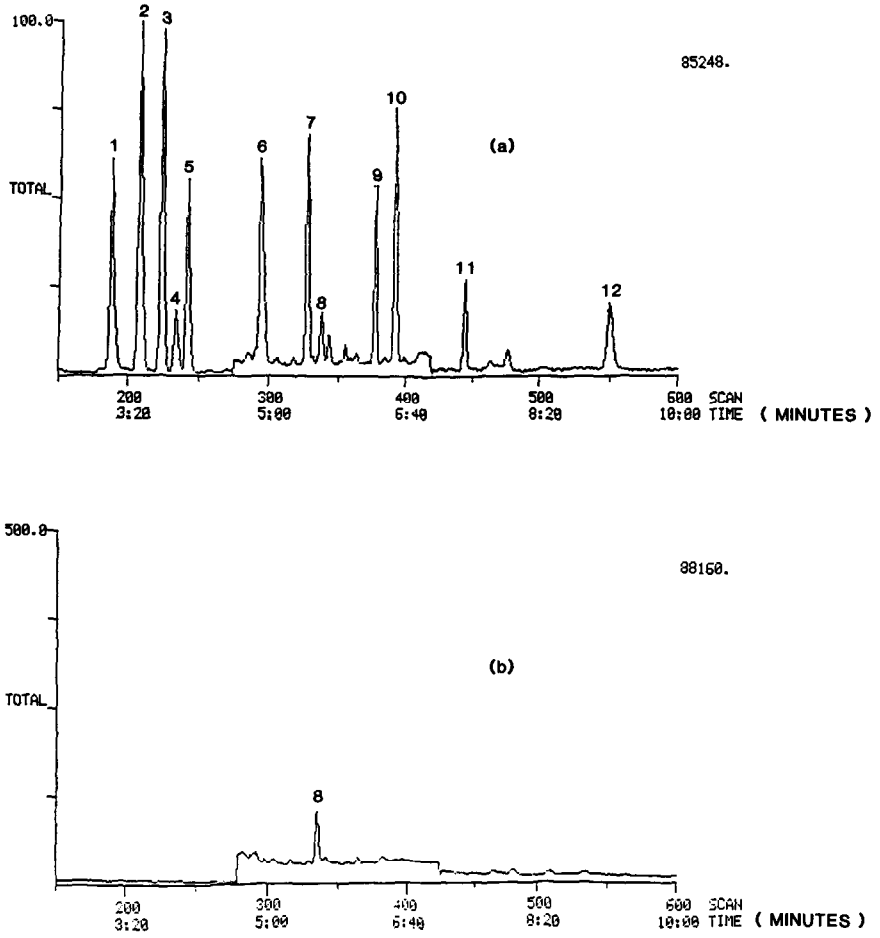

Fig. 2. Reconstructed total ion current chromatograms of (a) human blood (2 ml) spiked at the 10 ppb level with a standard mixture of eleven trichothecenes and (b) unspiked human blood (2 ml). The term "total ion" refers to those ions taken from three subsets of ions listed in Table I, and accounts for the steps on the baseline. Peaks: 1 = nivalenol; 2 = scirpentriol; 3 = deoxynivalenol; 4 = T-2 tetraol; 5 = verrucarol; 6 = 15-monoacetoxyscirpenol; 7 = 15-acetyl-deoxynivalenol; 8 = 7-hydroxy-DAS (internal standard); 9 = T-2 triol; 10 = diacetoxyscirpenol; 11 = HT-2; 12 = T-2.

higher. Routinely, the ion source was set at 60°C since this was the lowest controllable setting with the Finnigan 4510 instrument. This temperature dependence of the molecular ion to the *m/z* 163 ion ratio directly parallels reactions within an electron capture detector where associative capture is favoured by lower temperatures[33]. With the exception of T-2, DAS and 7-OH DAS, the base peak for all trichothecenes was the molecular ion M^-. In all cases additional characteristic peaks were observed at $(M - 20)^-$, corresponding to loss of hydrogen fluoride from the molecular ion. The $M^-/(M - 20)^-$ ratio was also temperature dependent, being greater at lower temperatures as would be expected. The variation of this ratio for T-2 tetraol and scirpenetriol over the source temperature range 60–100°C is shown in Fig. 4. In the case

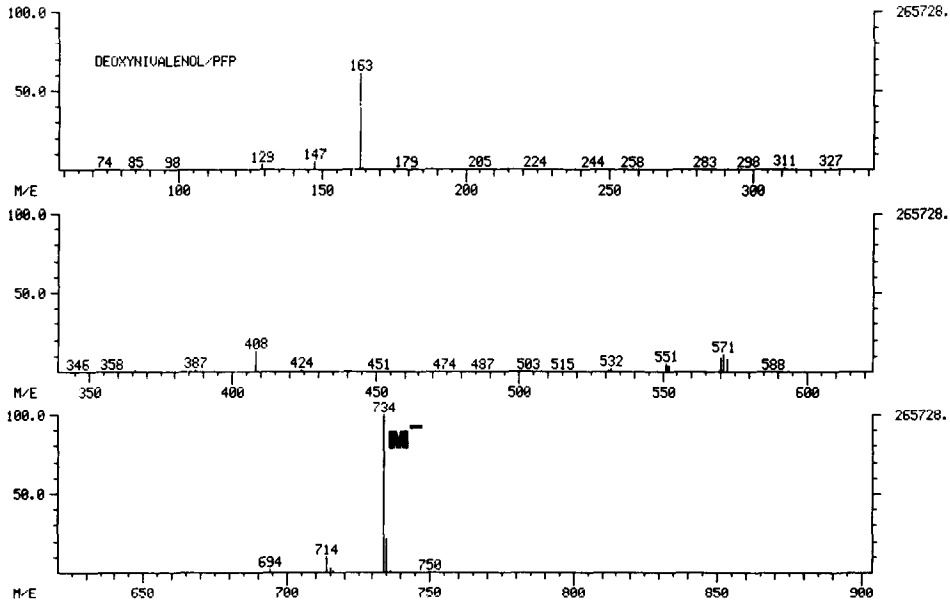

Fig. 3. ECNICI mass spectrum of the pentafluoropropionyl (PFP) derivative of deoxynivalenol at a source setting of 60°C.

of T-2, DAS and 7-OH DAS the base peak was always $(M - 82)^-$, followed by a peak at $(M - 20)^-$, the M^- being very weak, if observed at all. This loss of 82 could be attributed to the loss of ketene and hydrogen fluoride via an intramolecular rearrangement.

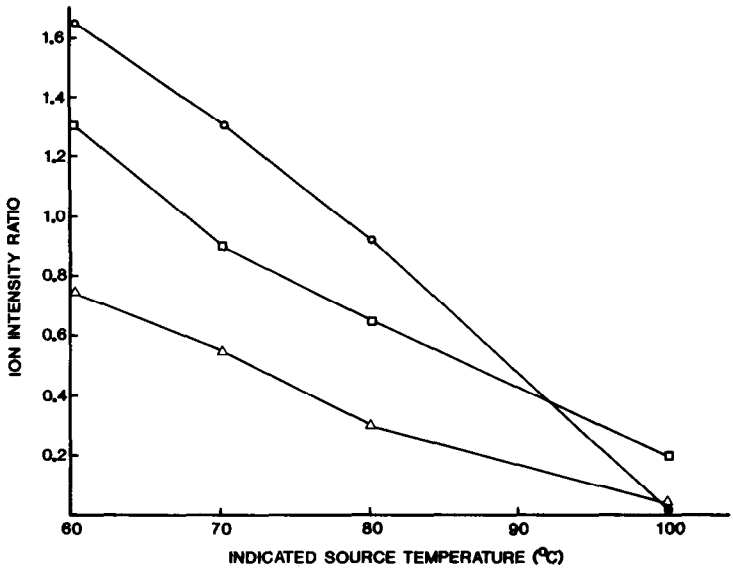

Fig. 4. The effect of the source temperature on some typical ion ratios. (△) HT-2, $(M^-)/(163^-)$; (○) T-2 tetraol, $(M^-)/(M-HF)^-$; (□) scirpentriol, $(M^-)/(M-HF)^-$.

The source pressure also affected the negative ion spectra albeit to a lesser degree than the source temperature. Raising the source pressure from the usual 0.3 Torr for chemical ionisation to 1.0 Torr increased both the total ion yield and the yield of the characteristic ions. For example, with T-2 tetraol, HT-2 and DAS at a source temperature of 60°C the yields of characteristic ions were increased by factors of 5, 3 and 6, respectively, by increasing the source pressure from 0.3 to 1.0 Torr. In addition, the ratio of the characteristic ion peaks changed. T-2 tetraol exhibited an $M^-/(M - 20)^-$ ratio of 0.97 at 0.3 Torr. This ratio increased to 1.96 at 1.0 Torr. Fragmentation of the molecular ion is therefore reduced at higher source pressures, presumably due to a third-body effect where more frequent collisions with non-reactive species will occur, thus dissipating excess vibrational energy within the molecular ion. The overall increased negative ion yield at higher source pressures is consistent with a greater efficiency of thermalisation of the electrons for subsequent capture.

The enhanced sensitivity afforded by ECNICI compared with positive ion chemical ionisation mass spectrometry is illustrated in Fig. 5 by pulsed positive ion negative ion chemical ionisation (PPINICI) spectra for T-2 tetraol. The total ion counts are over an order of magnitude higher, but equally important the ratio of the M^- to $(M + 1)^+$ peaks is even higher, the ratio being highest for T-2 tetraol, with the greatest number of electron capturing groups.

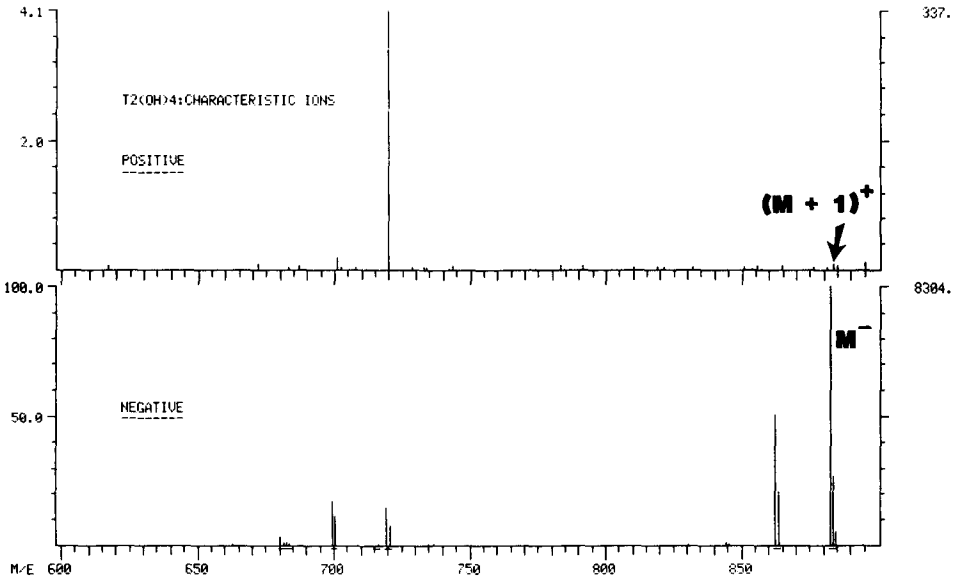

Fig. 5. PPINICI spectra for the pentafluoropropionyl derivative of T-2 tetraol using a source setting of 60°C. The ratio of the ion counts (8304:337) for the major characteristic negative (*m/z* 882) and positive (*m/z* 720) ions illustrate the enhanced sensitivity of ECNICI.

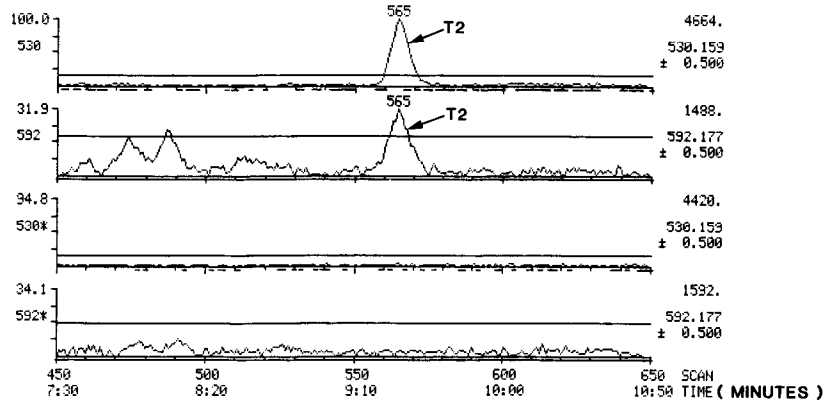

Fig. 6. Selected-ion chromatograms for *m/z* 530 and *m/z* 592 of a blood sample (2 ml) spiked with 10 ppb T-2 and of the corresponding system blank (lower two profiles); 10:1 signal-to-noise (S/N) thresholds are indicated on all four chromatograms.

Overall method

The detection limits obtained are shown in Table II for spiked solutions and for spiked blood which has been through the described clean-up procedure. Comparison of the first two columns in Table II affords the minimum extraction efficiency of the work-up, which is greater than 20% in all cases for samples spiked at the ppb level. The responses from spiked blood samples containing T-2 (10 ppb), DAS (2 ppb) and verrucarol (2 ppb) are shown in Figs. 6–8. The detection limits are quoted at a 5:1 signal-to-noise ratio and are those that were achievable routinely over several weeks. No interferences were observed in normal blood at these low levels, confirming the excellent selectivity achievable using negative ion techniques. The responses for the trichothecenes, normalized to the internal standard, were found to be linear over at least two orders of magnitude as illustrated in Fig. 9 for DON.

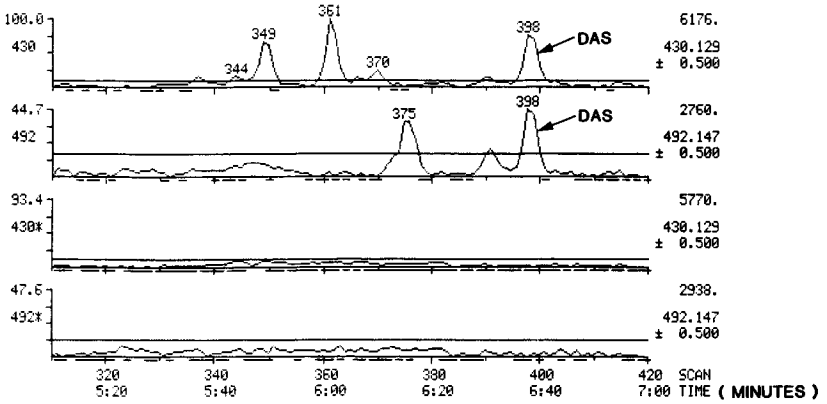

Fig. 7. Selected-ion chromatograms for *m/z* 430 and *m/z* 492 of a blood sample (2 ml) spiked with 2 ppb DAS and of the corresponding system blank (lower two profiles); 10:1 S/N thresholds are indicated on all four chromatograms.

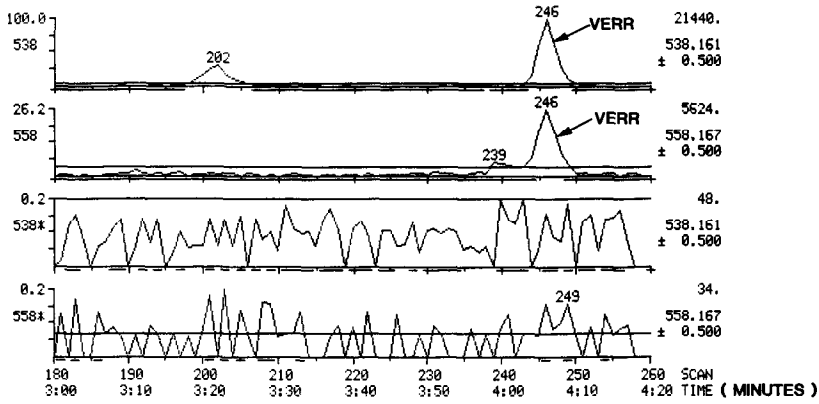

Fig. 8. Selected-ion chromatograms for *m/z* 538 and *m/z* 558 of a blood sample (2 ml) spiked with 2 ppb verrucarol and of the corresponding system blank; 10:1 S/N thresholds are indicated on all four chromatograms.

The overall procedure was assessed in collaborative studies in which two sets of spiked blood samples, 42 in all, including negative controls, were analysed. The results of our analyses are shown in Tables III and IV. Complete (100%) detection of spiked samples was achieved along with zero false positives, the latter being defined as finding trichothecenes in blank samples or in samples which do not contain a precursor capable of simple hydrolysis to the observed species. In the case of the HT-2 spikes in Table III, substantial hydrolysis to T-2 tetraol was observed, this

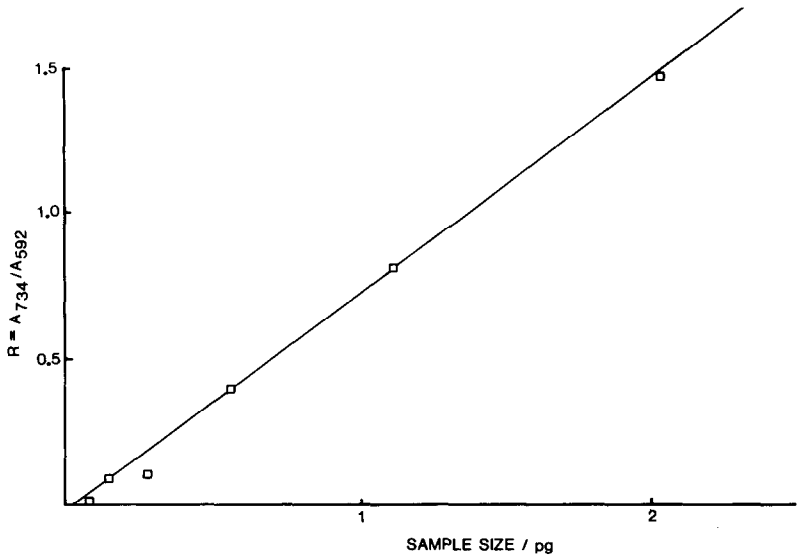

Fig. 9. Calibration graph (slope = 0.773; correlation = 0.993) for deoxynivalenol. A_{734} and A_{592} are the areas for the characteristic quantitation ions for deoxynivalenol (*m/z* 734) and internal standard (*m/z* 592) respectively.

TABLE III

ANALYSIS OF UNKNOWN SAMPLES (SET ONE)

Species	*Spike (ppb)*	*T-2 (ppb)*	*HT-2 (ppb)*	*T-2 tetraol (ppb)*
	0	–*	–	–
	0	–	–	–
	0	–	–	–
	0	–	–	–
T-2	7.5	5	–	–
	7.5	4	–	–
	75	60	–	–
	75	55	–	–
	100	220	–	–
	100	85	–	–
HT-2	7.5	–	–	10
	7.5	–	–	4
	75	–	4	8
	75	–	2–3	12
	150	–	15	10
	150	–	6	45
T-2 tetraol	7.5	–	–	3
	7.5	–	–	2
	75	–	–	200
	75	–	–	40
	150	–	–	4
	150	–	–	33

* – = Not found; T-2 triol, DAS, 15-MAS, S-3OL, NIV, DON and 15-AcDON were also not found.

being consistent with our own degradation studies, where HT-2 was observed to have an *in vitro* half-life of *ca.* 30 min in unstabilised heparinised blood at 37°C. By contrast, T-2 was more stable with a half-life of *ca.* 10 h under similar conditions[34]. All species listed in Fig. 1 were screened for but only the species included by the initiating laboratories were found except as noted for HT-2 spikes. This confirmed the value of using a vigorous protocol to avoid cross-contamination.

The quantitative reliability of the method was relatively poor, particularly for the more polar species such as nivalenol, verrucarol and T-2 tetraol, and is complicated by the unknown effects of both long term storage and blood stabilising additives on the stability of the individual trichothecenes. However, it was acceptable, since the primary objective of this work was the development of a screening method for the unambiguous and concurrent detection at *ca.* 1 ppb levels in blood of a range of trichothecenes with physicochemical properties ranging from those of the lipophilic T-2 toxins to the more hydrophilic toxins such as T-2 tetraol and nivalenol. This capability is particularly important, since as the collaborative study indicated, HT-2 undergoes extensive hydrolysis to T-2 tetraol even in blood samples which are stabilised and frozen. For more accurate quantitation a more restrictive clean-up procedure would be preferable, recovering a narrow range of trichothecenes and with

TABLE IV

ANALYSIS OF UNKNOWN SAMPLES (SET TWO)

	T-2 (ppb)	*NIV (ppb)*	*VERR (ppb)*	*DAS (ppb)*
Spiked level	200	150	10	10
Found*	400	250	6	12
Found	470	65	10	12
Found	380	360	6	9
Found	270	230	9	11
Spiked level	40	30	2	2
Found*	32	32	1	1
Found	33	42	1	1
Found	70	7	1	1
Found	33	75	1	1
Spiked level	10	10	150	200
Found*	8	40	160	220
Found	7	4	180	230
Found	11	7	250	250
Found	11	7	200	230
Spiked level	0	0	0	0
Found*	–**	–	–	–
Found	–	–	–	–
Found	–	–	–	–
Found	–	–	–	–
Spiked level	2	2	30	40
Found*	3	0.5	35	40
Found	2	0.5	1	40
Found	1	0.4	40	40
Found	4	0.4	7	42

* It subsequently emerged that the sample set consisted of four replicates of each of five standards.

** – = Not found; HT-2, T-2 triol, 15-MAS, S-3OL, DON, 15-AcDON and T-2 tetraol were also not found.

the addition of an internal standard suited to the physicochemical characteristics of a particular trichothecene. Furthermore, quantitation would be improved by optimising the derivatisation conditions and chromatography for specific trichothecenes.

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