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DETECTION OF TRACE LEVELS OF TRICHOTHECENE MYCOTOXINS IN HUMAN URINE BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

A method is described for the simultaneous detection of the trichothecene mycotoxins T-2, HT-2, T-2 tetraol, diacetoxyscirpenol, 15-monoacetoxyscirpendiol, scirpentriol, nivalenol and deoxynivalenol, in human urine. Samples were extracted from Clin Elut columns and cleaned up using reversed-phase Sep-Pak C_{18} cartridges. Trichothecenes were derivatised as their heptafluorobutyryl esters, and detected by gas chromatography–mass spectrometry–selected-ion monitoring using electron impact ionisation. The method was validated by the analysis of 22 urine samples, spiked and submitted "blind" for analysis by another laboratory. An alternative gas chromatography–mass spectrometry method using negative ion chemical ionisation is also described and a preliminary comparison of the two methods made. The methods enabled levels down to 1 ppb to be detected, with confirmation of identity at levels between 2 and 5 ppb, depending on the toxin.

INTRODUCTION

Trichothecene mycotoxins are a structurally related group of sesquiterpenoid secondary metabolites produced by species from several genera of fungi[1]. For many years trichothecenes have been of concern to the agricultural community because of widespread, though sporadic, contamination of cereal and other crops with trichothecenes produced by species of *Fusarium*. More recently it has been claimed that trichothecenes have been used as chemical warfare agents in Southeast Asia[2–5]. Though more than 60 trichothecenes have been isolated from laboratory cultures, relatively few, T-2, HT-2, diacetoxyscirpenol (DAS), neosolaniol, deoxynivalenol (DON) and nivalenol (NIV), have been detected in foodstuffs[1]. For example, deoxynivalenol has attracted particular attention because of its widespread occurrence in North American corn and wheat[6]. T-2 toxin, though occurring relatively rarely in cereal, was implicated[7,8] as the major causative agent through consumption of mouldy grain, of a devastating outbreak of alimentary toxic aleukia, a mycotoxicosis which caused thousands of human deaths in Siberia during the 1940s[9].

To confirm that a human exposure to trichothecenes has occurred, either by ingestion of contaminated food or through deliberate exposure, it is desirable to have

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specific and sensitive methods for the detection of these compounds in biological fluids. On pharmacokinetic grounds[10], for samples taken hours or days after an exposure, these methods should be capable of detecting trichothecenes and their possible metabolites at levels down to 1–10 ppb* (1–10 ng ml^{-1}) in blood or urine. In biological systems it is equally important to be able to detect the hydrolysis products, *e.g.* T-2 tetraol and scirpentriol, of esterified toxins such as T-2 and DAS, since these may be important metabolites *in vivo*[10,11], or be formed through chemical or enzymatic hydrolysis subsequent to sampling[12]. Our objective was therefore to be able to detect a range of trichothecenes and their hydrolysis products at levels down to 1–10 ppb in human urine. A gas chromatography–mass spectrometry (GC–MS) method used previously for the detection of trichothecenes in biomedical samples did not include this range of toxins[2].

Several GC–MS methods have been reported for the detection of trichothecenes, mostly for the analysis of grain samples. The majority have been restricted to a narrow selection of trichothecenes, often excluding the more polar ones T-2 tetraol, scirpentriol and nivalenol. These trichothecenes are generally the more difficult to recover. For example, Rosen and Rosen[13] reported a method for the detection of T-2, DAS and HT-2 in corn. Trichothecenes were derivatised as their trimethylsilyl (TMS) ethers and detected by selected-ion monitoring using electron impact (EI) ionisation. The combination of TMS derivatives and EI ionisation was used also by Gilbert *et al.*[14] and by Chaytor and Saxby[15] for the analysis of DON and T-2 respectively in grain, and by Yoshizawa and Hosokawa[16] for the detection of NIV and DON in foodstuffs. Mirocha *et al.*[2] used trifluoroacetate derivatives with EI or positive chemical ionisation (PCI) for the detection of T-2, HT-2 and DAS in blood and urine. Single-ion monitoring of heptafluorobutyryl (HFB) ester derivatives using EI ionisation was first reported by Scott *et al.*[17] as confirmation of the detection of DON by GC with electron-capture detection. An advantage of using HFB derivatives is that ions of higher mass can be used for selected-ion monitoring (SIM), which may give lower chemical background. More recently high sensitivity has been demonstrated for the detection of perfluoro ester derivatives using electron-capture negative ion chemical ionisation (NICI)[12,18]. Increased sensitivity may result from a number of factors, including greater efficiency of ionisation, minimal fragmentation of molecular and other high mass ions, and reduced chemical background. Rothberg *et al.*[18] reported high sensitivity using this technique for the detection of T-2, HT-2, neosolaniol, DAS, DON and fusarenon-X as their HFB derivatives in corn. A disadvantage to the use of HFB derivatives is that the tetra-HFB derivatives of T-2 tetraol and NIV give molecular ions above the upper mass limit m/z 1000 of many quadrupole mass spectrometers. Begley *et al.*[12] overcame this problem by using pentafluoropropionyl esters and NICI to detect a wide range of trichothecenes in blood. NIV and DON, which possess an enone function, may be detected as their TMS derivatives using NICI[19]. Thermospray LC–MS is a promising alternative to GC–MS for the detection of trichothecenes in biological fluids, but so far has not achieved the sensitivity which we required for the detection of T-2 tetraol[20].

The relative merits of using EI, PCI or NICI depend on the mass spectrometer

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

employed, particularly with regard to its source design. Preliminary studies at the Chemical Defence Establishment (CDE) using a VG7070EQ magnetic sector instrument, indicated that no useful increase in sensitivity could be achieved using PCI or NICI. In this paper we describe a GC–MS method, using EI ionisation, which enables a range of trichothecenes to be detected as their HFB derivatives, at levels down to 1–10 ppb in human urine. The validation of this methodology is demonstrated by reporting the analyses of 22 spiked urine samples, including negative controls, prepared for analysis by an independent laboratory. We also report a preliminary comparison with an alternative method employing a quadrupole mass spectrometer and NICI.

EXPERIMENTAL

Materials

DON was purchased from Myco-Lab (Chesterfield, MO, U.S.A.). Other trichothecenes were isolated and purified at CDE. Fisons "Distol" grade solvents and HPLC grade water were used. Clin Elut columns (1005) were made by Analytichem International (Harbor City, CA, U.S.A.). Sep-Pak C_{18} cartridges were purchased from Waters Assoc. (Northwich, U.K.). Heptafluorobutyrylimidazole (HFBI) was purchased from Lancaster Synthesis (Lancaster, U.K.) and redistilled before use. Twenty-two spiked urine samples (*ca.* 5 ml) including negative controls, were prepared at the US Army Medical Research Institute for Infectious Diseases (USAMRIID), Fort Detrick, Maryland, U.S.A. A computerised random number generator was used to code the samples, the master code known only to staff at USAMRIID. Samples were frozen after preparation, transported frozen by air to the U.K., and stored frozen (−20°C) for eight months prior to analysis.

Extraction and clean-up

Urine (3 ml) was absorbed onto a 5-ml Clin Elut tube. The tube was then eluted with ethyl acetate–methanol 95:5 (4 × 8 ml), the combined eluates dried with sodium sulphate (2 g), and concentrated to dryness in a 50-ml round-bottomed flask using a rotary evaporator. The residue was taken up in water (2 ml) and, using a syringe, loaded onto a Sep-Pak C_{18} cartridge. The round-bottomed flask 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 C_{18} cartridge. The 40:60 eluate containing T-2 tetraol, scirpentriol, NIV and DON was concentrated to dryness at 30–40°C in a 50-ml round-bottomed flask using a rotary evaporator. The 90:10 eluate was diluted to 50:50 with water and extracted by tumbling for 10 min with dichloromethane (2 × 5 ml) in a 15-ml screw-capped tube. After drying with sodium sulphate (2 g) the extracts were combined with the residue from the 40:60 eluate and concentrated to dryness. The dry residue was transferred to a 1-ml vial using methanol, and the methanol removed in a stream of nitrogen at 60°C.

Derivatisation

To the residues was added 7-hydroxy-DAS (20 ng) in methanol (20 μl) as internal standard. The methanol was removed in a stream of nitrogen and the residues heated with HFBI (20 μl) in toluene–acetonitrile 95:5 (200 μl) for 2 h at 60°C. After

cooling, the solution was washed by vortexing for 1 min with 5% sodium bicarbonate solution (500 μl), and then with water (2 × 500 μl). Aliquots (0.5 μl) were injected.

GC–MS analysis

Method A. A VG7070EQ mass spectrometer was coupled to an 11/250 data system, and equipped with a Dani 3800 gas chromatograph. The gas chromatograph was fitted with a BP 1 (SGE U.K.) 25 m × 0.2 mm I.D. fused-silica column, film thickness 0.25 μm, leading directly into the ion source. Helium at 15 p.s.i. was used as carrier gas. The oven was held at 160°C for 1 min, programmed from 160 to 275°C at 10°C min^{-1}, and held at 275°C for 5 min. Splitless injections (0.5 μl) were made; split delay, 0.5 min; septum purge, 1 ml min^{-1}; injector temperature, 260°C; transfer line temperature, 250°C.

The mass spectrometer was operated at nominal mass resolution in the SIM mode using EI ionisation at 70 eV, source temperature 220°C and trap current 200 μA. For screening purposes, two ions were monitored for each trichothecene as listed first in Table I. The mass spectrometer was programmed to monitor ions for T-2 tetraol, NIV, DON and scirpentriol between retention times 5.5 and 9.5 min, and for T-2, HT-2, DAS, 15-MAS and 7-OH DAS between 10 and 16 min. For confirmation of identification, up to four additional ions were monitored for each trichothecene as shown in Table I. The minimum criteria for a confirmed identification were at least three ions at the correct retention time (± 1%) with signal-to-noise (S/N) ratios greater than or equal to 3:1, and in ratios consistent with those of a standard[21].

Quantitation was determined by comparing computer integrated peak areas (*i.e.* ion counts) of the ions listed first in Table I, to that for the ion *m/z* 714 in 7-OH DAS, added as internal standard before derivatisation[12]. Linear calibration curves were established for T-2, HT-2 and T-2 tetraol spiked into urine at concentrations of 2, 5, 10, 20, 35, 50 and 100 ppb. For the analysis of unknown samples, a sample of urine spiked at 20 ppb was run with each batch of three unknowns to assure system sensitivity and provide response factors for quantitation. Mandatory glassware blanks were run as controls before each unknown sample, to ensure freedom from cross contamination.

Method B. Detection employing a Finnigan 4600 GC–MS system used a modification of the method developed by Begley *et al.*[12] for the analysis of blood samples.

TABLE I

IONS USED FOR SELECTED ION MONITORING (EI)

Trichothecene–HFB	*m/z*
T-2	518, 501; 578, 560, 457, 427
HT-2	655, 672; 732, 714, 654, 641
T-2 tetraol	869, 868; 870, 656, 655, 654
DAS	502, 474; 459, 442, 429, 415
15-MAS	656, 628; 673, 657, 643, 641
Scirpentriol	870, 855; 657, 656, 643, 628
NIV	1096, 1077; 883, 882, 865, 853
DON	884, 865; 687, 671, 640, 615
7-OH DAS	714, 671

TABLE II

IONS USED FOR SELECTED-ION MONITORING (NICI)

Trichothecene–HFB	*m/z*
T-2	580, 642
HT-2	816, 796
T-2 triol	970, 950
T-2 tetraol	1082, 1062
DAS	480, 542
15-MAS	716, 696
Scirpentriol	870, 850
NIV	1096, 1076
DON	884, 864
7-OH DAS	692, 754

The gas chromatograph was fitted with a BP5 (SGE) 12 × 0.22 mm I.D. fused-silica column, film thickness 0.25 μm. Helium at 8 p.s.i. was used as carrier gas. The oven was held at 90°C for 2 min, programmed from 90 to 180°C at 20°C min^{-1}, 180 to 240°C at 5°C min^{-1} and held at 240°C for 2 min. Splitless injections (0.5 μl) were made; split delay, 0.6 min; septum purge, 2 ml min^{-1}; injector temperature, 300°C; transfer line temperature, 250°C.

The mass spectrometer was operated in the SIM mode using NICI with methane as reagent gas. The source was operated at a dial reading of 60°C; filament voltage, 100 eV; emission current, 0.3 mA; source pressure, 0.8 Torr. Pairs of ions were monitored for each trichothecene as shown in Table II: those for NIV, scirpentriol, DON and T-2 tetraol between retention times 6 and 9 min; 15-MAS, 7-OH DAS, T-2 triol and DAS between 9 and 12.5 min; and HT-2 and T-2 between 12.5 and 18 min. Quantitation was performed as above. Linear calibration curves were established for T-2, HT-2 and T-2 tetraol as described above.

RESULTS AND DISCUSSION

The recovery of the range of trichothecenes of interest, in a single screening procedure, is complicated by the wide variation in physicochemical and chromato-

TABLE III

RECOVERY OF TRICHOTHECENES (20 ppb, 20 ng ml^{-1}) FROM URINE

Trichothecene	*Recovery (%)**	*Coefficient of variation (%)*
T-2	55	16
HT-2	72	18
T-2 tetraol	36	3
DAS	65	17
15-MAS	84	7
Scirpentriol	59	2
NIV	42	9
DON	57	18

* Average of three separate determinations.

graphic properties. T-2 tetraol and nivalenol, the most hydrophilic of the toxins investigated, are poorly extracted from urine by water immiscible solvents, and are also inefficiently retained by XAD-2 resin. One solution to this type of problem is to use disposable extraction columns such as Clin Elut, which facilitate extraction by absorbing and distributing the aqueous phase over a large inorganic surface. Ethyl acetate alone as extracting solvent gave poor recoveries (less than 10%) of T-2 tetraol, scirpentriol and nivalenol from Clin Elut tubes. Recoveries were improved by adding increasing proportions of methanol to the solvent, but at the expense of extracting additional extraneous material. Chloroform–methanol 95:5 provided a suitable compromise, using four volumes of solvent for extraction rather than two. Further clean-up of the extracts was performed on reversed-phase Sep-Pak C_{18} cartridges[12]. The non-esterified trichothecenes (T-2 tetraol, scirpentriol, NIV and DON) were eluted with methanol–water 40:60, and the partially esterified ones (T-2, HT-2, T-2 triol, DAS and 15-MAS) eluted with methanol–water 90:10. The latter were then extracted into dichloromethane prior to concentration, to avoid any hydrolysis which was sometimes observed on concentrating solutions in aqueous methanol. The recoveries for urine spiked at 20 ppb, shown in Table III, were sufficient over the range (36–84%) to achieve the detection limits required of the assay. Reproducibility was acceptable for a screening procedure for the simultaneous detection of such different analytes, and without the addition of internal standards to compensate for variation in recovery.

Under the conditions employed (HFBI, 60°C, 120 min) nivalenol formed two tetra-HFB derivatives, the one with the longer retention time predominating in a ratio of *ca.* 6:4, as determined on a standard using GC with flame ionisation detection. Both derivatives had a molecular weight of 1096 as indicated by their ammonia PCI mass spectra. These probably result from isomerisation of the C-8 keto and C-7 hydroxy functions. The derivative with the shorter retention time, which predominated under very mild derivatising conditions, was formed to a much lesser extent in derivatised extracts of spiked urine (Fig. 1). Derivatisation of nivalenol at 100°C for 150 min gave only the second tetra-HFB derivative, but these more vigorous

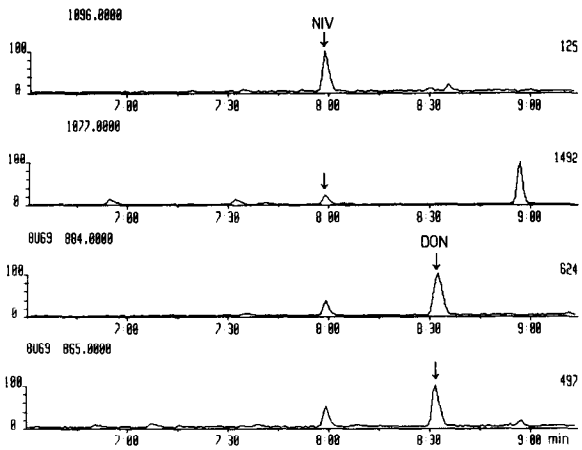

Fig. 1. Selected-ion current profiles for NIV and DON spiked into urine at 20 ppb.

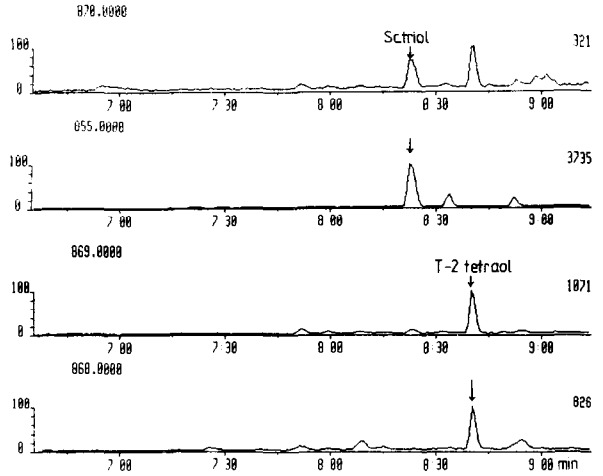

Fig. 2. Selected-ion current profiles for scirpentriol and T-2 tetraol spiked into urine at 20 ppb.

conditions adversely affected the HFB derivatives of T-2 and DAS. The conditions employed were therefore a compromise with respect to nivalenol, and accurate quantitation would best be performed in a separate determination. 7-OH DAS required heating for 90 min at 60°C to affect complete derivatisation, and DON for 60 min at 60°C[17]. The other trichothecenes could be fully derivatised at room temperature.

Figs. 1–4 show selected-ion current profiles for trichothecenes spiked into urine at 20 ppb, monitoring two ions each for NIV, DON, scirpentriol, T-2 tetraol, DAS, HT-2, T-2 and 15-MAS. Note that the data system has normalised responses to the most intense peak in each chromatogram. Fig. 5 shows confirmation of the detection of HT-2 toxin at this concentration, monitoring six ions. Sensitivity was generally greater for the polyhydroxy trichothecenes, shown by the excellent S/N ratios observed in the selected-ion current profiles for T-2 tetraol spiked into urine at 2 ppb

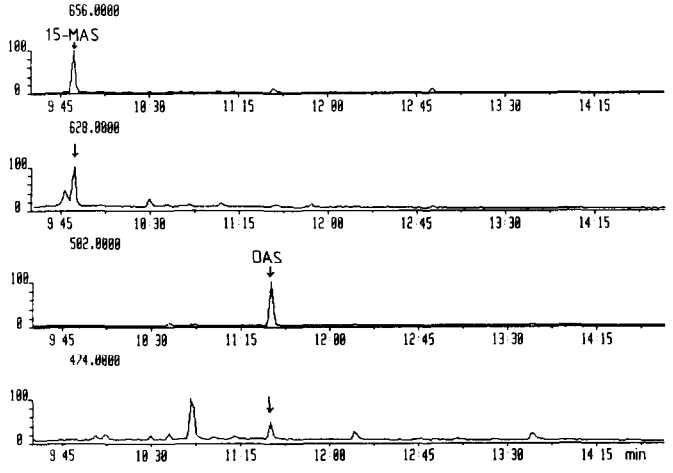

Fig. 3. Selected-ion current profiles for 15-MAS and DAS spiked into urine at 20 ppb.

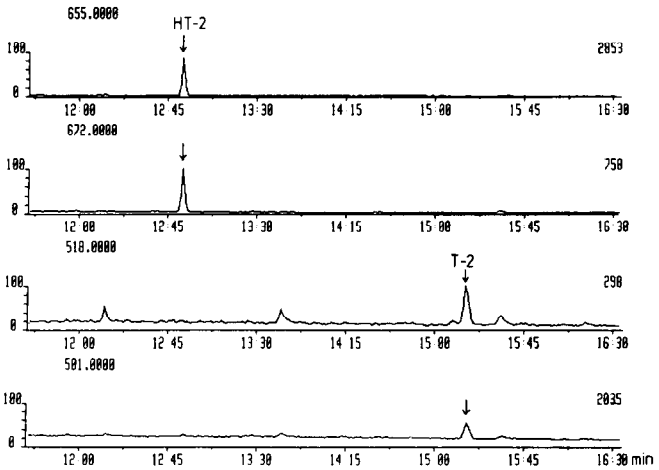

Fig. 4. Selected-ion current profiles for HT-2 and T-2 toxins spiked into urine at 20 ppb.

(Fig. 6). Limits of detection for the eight trichothecenes, based on S/N ratios less than or equal to 3:1 for the most intense ions, varied from 1 ppb for T-2 tetraol to 2–5 ppb for T-2 toxin. Confirmation of identity with at least three ions could be achieved down to levels of 2–7 ppb. Calibration curves for T-2, HT-2 and T-2 tetraol were linear over the range 0–100 ppb. In a few urine samples we have observed a

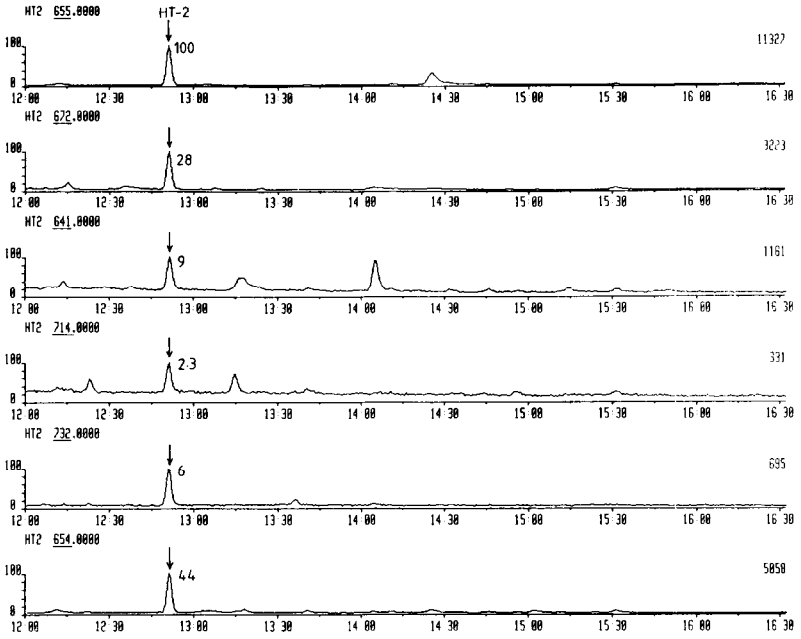

Fig. 5. Selected-ion current profiles for HT-2 toxin spiked into urine at 20 ppb, monitoring six ions for confirmation of identity.

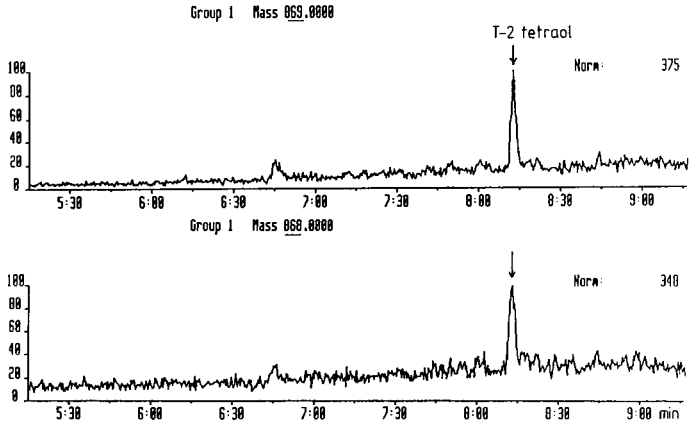

Fig. 6. Selected-ion current profiles for T-2 tetraol spiked into urine at 2 ppb.

TABLE IV

ANALYSES OF UNKNOWN SPIKED URINE SAMPLES

	Spike (ppb)	*T-2 (ppb)*	*HT-2 (ppb)*	*T-2 tetraol (ppb)*
	0	–*	–	–
	0	–	–	–
	0	–	–	–
	0	–	–	–
T-2	7.5	7	–	–
	7.5	10	–	–
	75	36	–	–
	75	108	–	–
	150	60	–	–
	150	121	–	–
HT-2	7.5	–	6	–
	7.5	–	8	–
	75	–	66	–
	75	–	36	–
	150	–	88	–
	150	–	74	–
T-2 tetraol	7.5	–	–	6
	7.5	–	–	4
	75	–	–	60
	75	–	–	30
	150	–	–	68
	150	–	–	66

* – = not found; DAS, 15-MAS, scirpentriol, NIV and DON also not found.

compound with a retention time similar to that of DON, and which gave responses equivalent to 2–5 ppb. It could be distinguished from DON on the basis of ion ratios.

The effectiveness of the method was tested in a collaborative study, in which 22 unknown samples were analysed. The samples were transported frozen over dry ice by air, and stored frozen for eight months prior to analysis. Analyses for the eight trichothecenes described above, together with the levels as spiked, are shown in Table IV. No false positives or false negatives were obtained. The trichothecenes T-2, HT-2 and T-2 tetraol were detected qualitatively as spiked, and no hydrolysis of T-2 or HT-2 to T-2 tetraol was observed. Each sample analysis was accompanied by a negative glassware control. We regard this as mandatory for trace analysis. Quantitation of samples spiked at 7.5 and 75 ppb was good in most cases but the higher 150 ppb levels were underestimated. This was not unexpected since response factors were made daily against samples spiked at 20 ppb; samples spiked at 100 ppb would have been more appropriate for the higher concentrations. The quantitative effects of transportation and prolonged storage are unknown, and some degradation or adsorption onto sample vials may have occurred. The analyses do however demonstrate the effective preservation of samples stored for many months at −20°C. Fig. 7 shows the detection of T-2, HT-2 and T-2 tetraol in unknown samples spiked at 7.5 ppb. All could be confirmed on at least four ions as shown for T-2 toxin in Fig. 8.

More recently we have compared the sensitivity of the method described above, with that obtainable using NICI and a quadrupole mass spectrometer. As discussed elsewhere[12], excellent sensitivity can be achieved using NICI and perfluoro ester derivatives, provided the source temperature is kept sufficiently low to prevent the

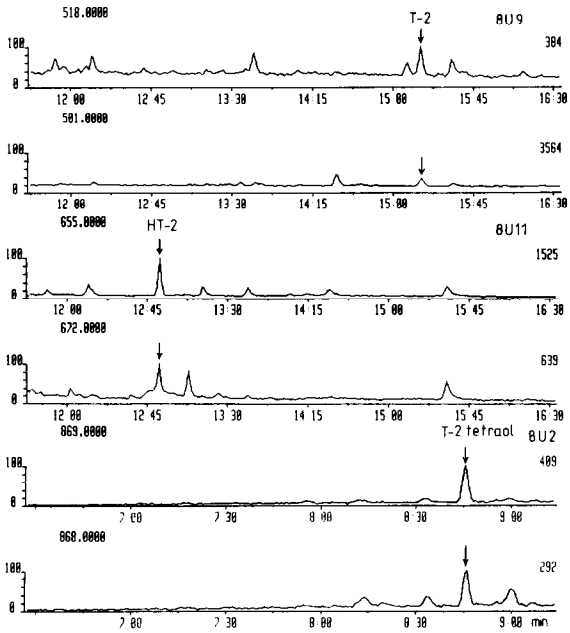

Fig. 7. Selected-ion current profiles for three unknown urine samples (coded 8U9, 8U11 and 8U2), found to contain respectively T-2 toxin (7 ppb), HT-2 toxin (6 ppb) and T-2 tetraol (6 ppb). Samples were spiked at 7.5 ppb levels.

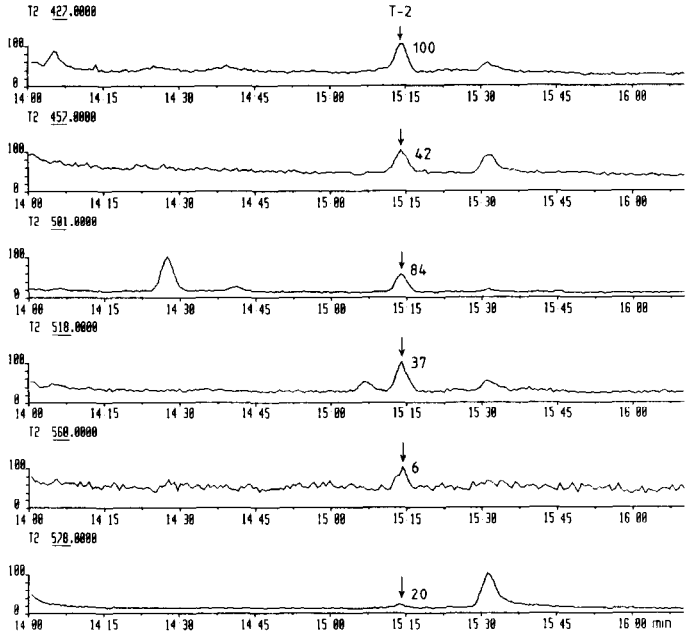

Fig. 8. Selected-ion current profiles for unknown urine sample (8U9), monitoring six ions for confirmation of identity of T-2 toxin (6 ppb). Ion ratios are shown.

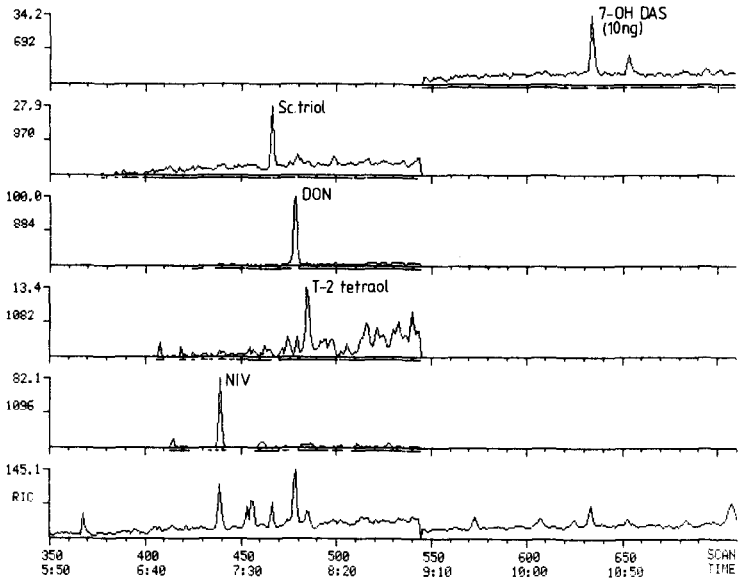

Fig. 9. Selected-ion current profiles and reconstructed ion current, using NICI, for scirpentriol, DON, T-2 tetraol and NIV spiked into urine at 5 ppb. 7-OH DAS is added as internal standard.

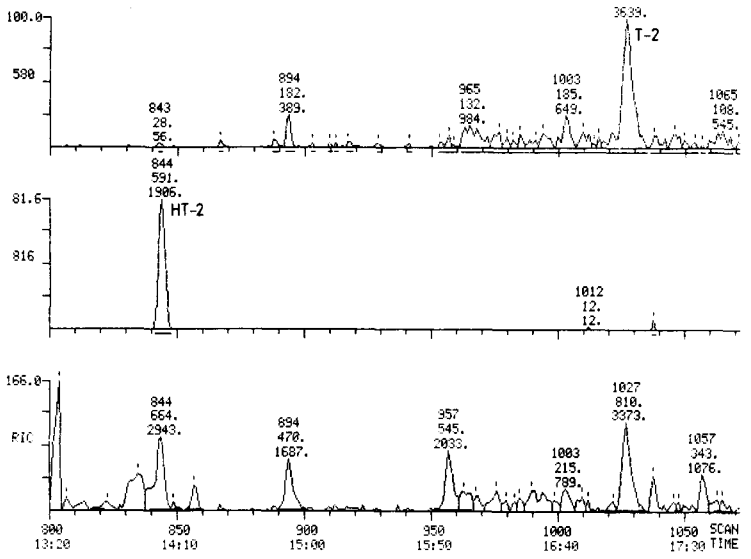

Fig. 10. Selected-ion current profiles and reconstructed ion current, using NICI, for T-2 and HT-2 toxins spiked into urine at 2 ppb.

non-characteristic ion $CF_3(CF_2)_nCO_2^-$ dominating the spectrum. Under these conditions M^-, $[M - HF]^-$ or $[M - 82]^-$ are the major characteristic ions with few additional fragment ions. Fig. 9 shows selected-ion current profiles for the M^- ions of derivatised NIV, DON, T-2 tetraol and scirpentriol spiked into urine at 5 ppb. Under the conditions employed, some enhanced sensitivity was observed for detecting NIV and DON, but S/N ratios for T-2 tetraol were poorer. The method provided a useful increase in sensitivity for detecting T-2 and HT-2 toxins. Fig. 10 shows selected ion current profiles detecting T-2 and HT-2 spiked into urine at 2 ppb. This level of T-2 would have been very close to the limit of detection using EI ionisation. Linear calibration curves were established over the range 0–100 ppb for T-2, HT-2 and T-2 tetraol. Further comparative studies would be needed to accurately compare the two methods, but combined they allow the range of trichothecenes studied to be detected in urine at levels less than or equal to 1 ppb without confirmation of identity or 2–5 ppb with confirmation. In our hands, both methods gave limits of detection up to an order of magnitude lower than could be achieved using TMS derivatives, particularly for the more polar trichothecenes.

The work described was performed using normal urine samples to which the compounds of interest were added. In the case of trichothecenes taken systemically, it may be that they and their metabolites will be excreted to a significant degree as conjugates. The extent to which trichothecenes are excreted as glucuronide conjugates in primate species is unknown. Preliminary observations in other species[11,22], and *in vitro*[23], suggest that glucuronides may be important. If so then it should be relatively easy to incorporate an enzymatic hydrolysis prior to extraction.

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