Journal of Chromatography, 283 (1984) 223–230
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 16,254

QUANTIFICATION AND CONFIRMATION OF FOUR *FUSARIUM* MYCOTOXINS IN CORN BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY–SELECTED ION MONITORING

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(First received August 4th, 1983; revised manuscript received August 23rd, 1983)

SUMMARY

A rapid method for the simultaneous determination of T-2 toxin, HT-2 toxin, diacetoxyscirpenol and zearalenone has been developed. Corn samples (10 g) are extracted with methanol, defatted with hexane and subsequently cleaned-up using both reversed-phase (C_{18}) and normal-phase (silica gel) Sep-Pak cartridges. Confirmation of identity is made by gas chromatography–mass spectrometry–selected ion monitoring of three ions characteristic of the trimethylsilyl derivatives of the myocotoxins. Use of deuterated internal standards makes the method quantitavely reliable and increases sensitivity. Confirmation of identity as well as quantitation can be achieved at levels of *ca.* 20–50 ppb**, depending on the mycotoxin. Detection limits (without confirmation of identity) are estimated at 1–20 ppb. Recoveries at the 46–111 ppb level ranged from 80 to 103% with coefficients of variation ranging from 1.6 to 14.2%.

INTRODUCTION

The trichothecenes are a structurally related group of sesquiterpenoid compounds produced by several genera and species of imperfect fungi[1]. Over 60 trichothecenes have been isolated and characterized, but only a few (T-2 toxin, diacetoxyscirpenol, nivalenol and deoxynivalenol) have ever been found to occur naturally in foodstuffs[2]. Recently, HT-2 toxin has also been reported to occur naturally in both wheat and maize[3]. Zearalenone, a mycotoxin with estrogenic properties and structurally unrelated to the trichothecenes, is also often found in food and feed samples contaminated with fusaria. The trichothecenes have received world-wide attention in the recent past because several of them are apparently ingredients of "Yellow Rain", a chemical warfare agent allegedly in use in Southeast Asia since 1975[4,5].

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

Scott[6] reviewed 52 publications dealing with trichothecene methodology. Using the following criteria – method published in full, greater than 70% recovery (with coefficient of variation less than 30%, if determined), reported detection limit below 100 ng/g grain for at least one of the trichothecenes – Scott singled out eight mehtods for attention. Of these, only three were also capable of confirmation of identity – the single ion detection methods of Scott *et al.*[7] and Collins and Rosen[8], and the radioimmunoassay method of Lee and Chu[9]. The latter two suffered from inability to detect more than one mycotoxin. The former method was unable to confirm the identity of T-2 toxin at or below 500 ppb. In addition, many would argue that the procedures of Scott *et al.* as well as those of Collins and Rosen are not truly confirmative because only a single ion is monitored.

Since publication of Scott's review[6], four papers on this subject have been published. Cohen and Lapointe[10] described a capillary gas–liquid chromatography (GLC) method for determination of deoxynivelenol in wheat and barley with a limit of detection of 50 ppb. Unequivocal confirmation of identity was achieved by single ion detection at 10,000 resolution. A method for five *Fusarium* mycotoxins with unequivocal confirmation of identity based on three selected ions was published by Trenholm *et al.*[11] but no recovery, sensitivity or precision data were given. Bata *et al.*[3] reported a method for six *Fusarium* mycotoxins based on capillary GLC, but this method is not confirmative. Rosen and Rosen[12] used three selected ions for confirmation of identity and quantitation of four mycotoxins in "Yellow Rain", but no recovery data was given because the nature of the substrate analyzed was unknown. Chaytor and Saxby[13] described a rapid and sensitive selected ion monitoring method for T-2 toxin in corn using two ions for confirmation.

We report a method for the analysis of three trichothecene mycotoxins and zearalenone in corn. Our criteria are more stringent than those recommended by Scott[6], because we include confirmatory capability in the method and demand 100 ppb or better sensitivity for all the mycotoxins. In addition, the method is more rapid and less cumbersome than most, if not all, published methods.

EXPERIMENTAL

Gas chromatography–mass spectrometry

A Finnigan Mat 311A mass spectrometer, which was interfaced by an all-glass SGE Scientific jet separator to a Varian 3700 gas chromatograph, was used for the analyses. The gas chromatograph was equipped with a 1 m × 2 mm I.D. glass column packed with 3% OV-17 on Chromosorb W HP (100–120 mesh) and was operated as follows: injection port and detector temperature, 310°C; helium carrier gas flow-rate, 25 ml/min; column temperature programmed from 180–260°C at 12°C/min and held at the final temperature for 5 min.

Conditioning of the glass surfaces with a minimum of five injections of 1 μg of trimethylsilyl T-2 toxin was necessary to assure low nanogram sensitivity for this material. The mass spectrometer conditions were: filament voltage, 100 eV; filament current, 2mA; glass jet and ion source temperatures, 310°C; resolution, 550; multiplier voltage, 2.4 kV (equivalent to a gain of *ca.* 5×10^6). Computerized selected ion monitoring was accomplished via the SS-200 data system. Three ions were monitored for each mycotoxin and two ions each were monitored for the T-2 toxin and diacetoxy-

scirpenol internal standards. The mass spectra of the trimethylsilyl mycotoxin derivatives, as well as the assignments for the ions monitored were published previously[12].

Materials and reagents

All solvents were Burdick & Jackson "High Purity" grade. N,O-Bis-(trimethylsilyl trifluoroacetamide (BSTFA) was purchased from Supelco. [$^2H_{18}$]-N,O-Bis(trimethylsilyl)-acetamide was purchased from Merck. The C_{18} reversed-phase and silica gel normal-phase Sep-Paks were purchased from Waters Assoc. All mycotoxins were purchased from Sigma. Field corn was obtained from Delaware Valley Cooperative and was, by analysis, found to be free of mycotoxins (limit of detection, 1–20 ppb).

Extraction and separation

Corn samples (10 g) were ground for 3 min in a Waring blender containing 50 ml of methanol and a 1-ml solution of mycotoxins to give the concentrations listed in Table I. The extraction procedure was repeated three times. Filtration of the methanol was accomplished by passing the slurry through a Buchner funnel containing diatomaceous earth. The methanol was extracted three times with 75 ml of hexane. The methanol solution was then evaporated to dryness, first with a rotary evaporator, and finally under a stream of nitrogen at 60°C. Methanol (2 ml) was added to redisolve the residue and once dissolved, 8 ml of distilled water were added. This 20% methanol solution was passed through a C_{18} reversed-phase "Sep-Pak" and the eluate discarded. Another 10 ml of 20% methanol was used to wash the vial and this solution was also passed through the Sep-Pak and discarded. (From a previous study[12] we know that the 20% methanol eluate would contain both deoxynivalenol and nivalenol had they been spiked into the corn. For further elaboration, see Discussion.) The four mycotoxins were then eluted from the Sep-Pak with 10 ml of methanol–water (85:15). The resulting eluate was evaporated under nitrogen (60°C). The residue was then dissolved in 0.5 ml toluene–ethyl acetate (1:1) and placed on the head of a silica gel normal-phase Sep-Pak. Another 10 ml of toluene–ethyl acetate was used to rinse the vial and this solution was also passed through the cartridge. The eluate was placed in a vial and evaporated to dryness under nitrogen at room temperature.

Derivatization

The final eluate was placed in a conical vial (Kontes) with 100–200 μl of ethyl acetate and 1 ml of BSTFA. The vial was capped and heated for 1 h at 90°C. The derivatized solution was evaporated in a stream of nitrogen at room temperature and

TABLE I

IONS USED FOR QUANTIFICATION OF MYCOTOXINS

Mycotoxin	*m/z**
Diacetoxyscirpenol	378 (387)
HT-2 toxin	466 (484)
T-2 toxin	436 (445)
Zearalenone	429 (480)

* Ions in parentheses are for the corresponding deutero-derivatized internal standards.

the residue dissolved in 50 μl of an ethyl acetate solution containing 100.1 ng/μl of the [$^2H_9$]trimethylsilyl derivatives of T-2 toxin and 25.2 ng/μl diacetoxyscirpenol. Both standards had been prepared previously by derivatization with [$^2H_{18}$]-N,O-bis(trimethylsilyl)acetamide. The deuterated analogues of the trimethylsilyl derivatives of HT-2 toxin and zearalenone (50 μl of 31.5 and 27.2 ng/μl, respectively) were added after quantification/confirmation of T-2 toxin and diacetoxyscirpenol and confirmation of HT-2 toxin and zearalenone for reasons explained in Discussion.

Gas–liquid chromatography–selected ion monitoring

Confirmation of identity for all four mycotoxins was obtained by comparison of three ions for each mycotoxin as described previously[12]. A 2–5 μl injection of the solution containing the derivatized mycotoxins together with the deutero-derivatized T-2 toxin and deutero-derivatized diacetoxyscirpenol was made and the following ions were monitored: 378, 250 and 290 for derivatized diacetoxyscirpenol; 387 and 359 for deutero-derivatized diacetoxyscirpenol; 467, 466 and 347 for derivatized HT-2 toxin; 436, 350 and 290 for derivatized T-2 toxin; 445 and 359 for deutero-derivatized T-2 toxin; 462, 445 and 350 for derivatized zearalenone. The computer was programmed to monitor the diacetoxyscirpenol ions between retention time 3 and 5 min and the other ions were monitored between 5 and 8 min.

Quantification of the derivatized mycotoxins was made by comparing their computer-integrated peak areas to those of their corresponding deutero-derivatized internal standards. For quantification, only one ion of the derivatized mycotoxin and one ion of its deutero-derivatized analogue were compared (Table I).

It is important to perform the zearalenone quantification as rapidly as possible because the zearalenone derivative begins to deteriorate appreciably after an hour. The derivatives of the other mycotoxyns are stable at freezer temperatures for months.

RESULTS

Table II summarizes per cent recovery and per cent coefficient of variation (C.V.) obtained from four analyses of corn samples spiked with diacetoxyscirpenol, HT-2 toxin, T-2 toxin and zearalenone. All four analyses gave three-ion ratios consistent with those obtained in the absence of corn matrix for each of the mycotoxins. Excellent recoveries (91–103%) and precision (C.V., 1.6–8.7%) were obtained for all the mycotoxins except for T-2 toxin. The latter gave an 80% recovery with a 14.2% coefficient of variation.

TABLE II

RECOVERY OF MYCOTOXINS ADDED TO CORN*

Mycotoxin	*Level added (ng/g)*	*Level found (ng/g)*	*Recovery (%)*	*Coefficient of variation (%)*
Diacetoxyscirpenol	64	66	103	1.6
HT-2 toxin	46	42	91	8.7
T-2 toxin	111	89	80	14.2
Zearalenone	52	50	96	5.2

* Average of four separate determinations.

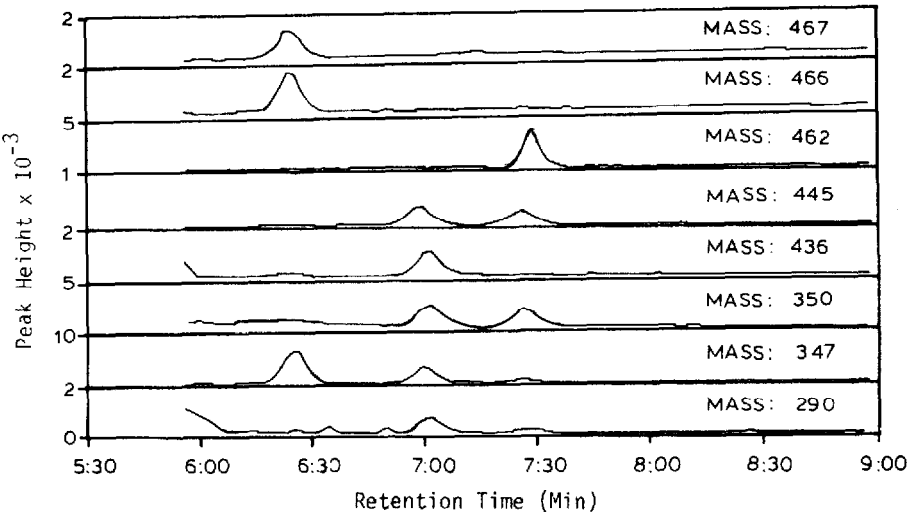

Fig. 1. Selected ion monitoring plot for confirmation of HT-2 toxin, T-2 toxin and zearalenone.

Fig. 1 is a computer print-out showing the three-ion confirmation of HT-2 toxin (*m/z* 467, 466, 347), T-2 toxin (*m/z* 436, 350, 290) and zearalenone (*m/z* 462, 445, 350) at retention times of 6:26, 7:02 and 7:26 min, respectively. The 445 ion at retention time 6:57 min is from the deuteroderivatized T-2 toxin and serves as an internal standard. The 359 ion from this material is not shown because the computer can print only eight ions at a time although it can store information from sixteen ions at each of seven retention time windows. Fig. 2 shows the three-ion confirmation of diacetoxyscirpenol at 4:39 min (*m/z* 378, 350 and 290) and its internal standard at 4:36 min (*m/z* 387, 359). Both figures represent the same corn sample. In some corn samples, the interference at 4:26 min for *m/z* 290 became too large for proper three-ion confirmation of diace-

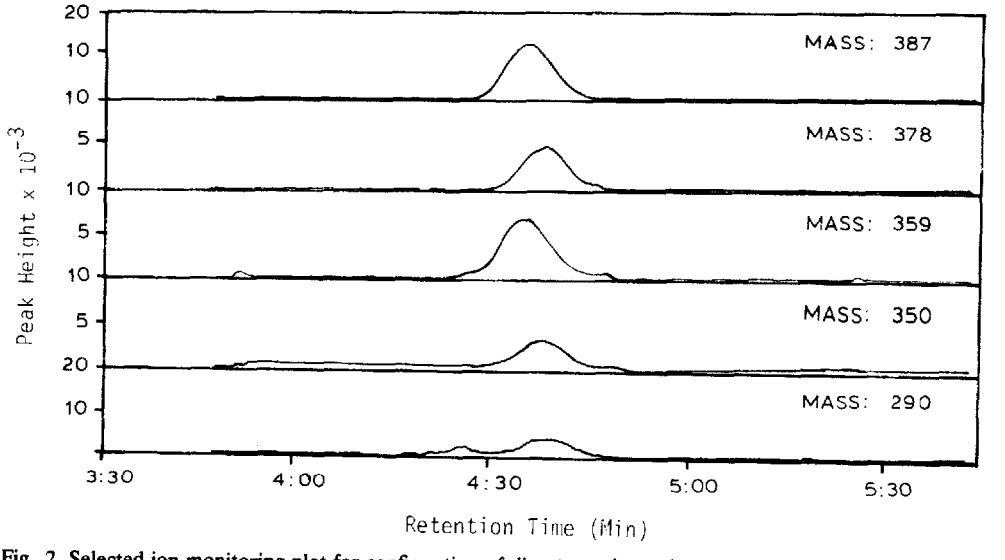

Fig. 2. Selected ion monitoring plot for confirmation of diacetoxyscirpenol.

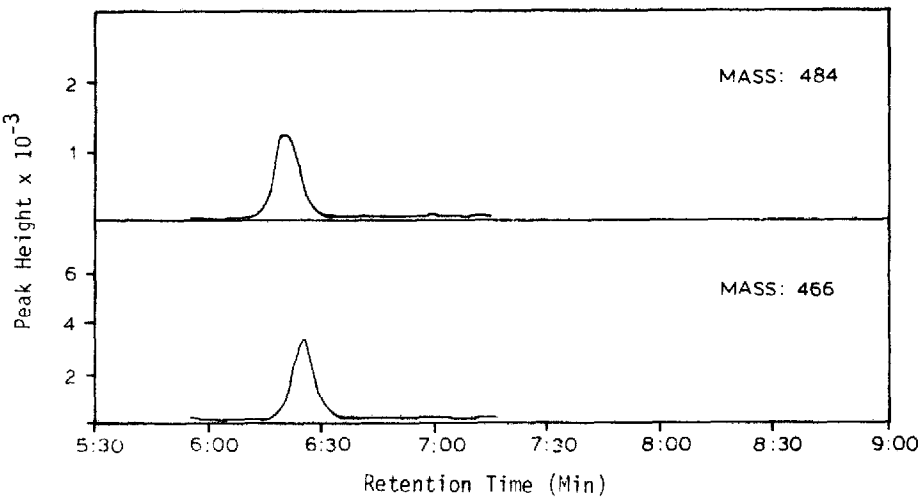

Fig. 3. Selected ion monitoring plot for quantification of HT-2 toxin.

toxyscirpenol. In such cases, the ion at *m/z* 379 was substituted with no loss in sensitivity. Fig. 3 shows quantification of HT-2 toxin at the 46 ppb level.

The area counts for the peaks in Figs. 1 and 2 range from a low of 3.97×10^3 for the 436 ion of T-2 toxin to a high of 3.97×10^4 for the 347 ion of HT-2 toxin. Since the data system can accurately integrate peaks with 1000-count areas, we conservatively estimate that confirmation of identity can be achieved at the 20 ppb level for diacetoxyscirpenol, HT-2 toxin and zearalenone and at *ca.* 50 ppb for T-2 toxin.

DISCUSSION

Analysis of food samples for toxins at the parts per billion level can result in misidentifications because of co-elution of materials with chromatographic behavior similar to those of the toxins. For example, Scott *et al.*[7] found that two of six wheat samples that tested positive for diacetoxyscirpenol by electron-capture detection were, in fact, false positives. A false positive for HT-2 toxin was also observed[7]. It is therefore of great importance that confirmation of toxin identity also be obtained, so that food of considerable economic and nutritional value not be unnecessarily lost. The method we report is designed to not only quantify some *Fusarium* mycotoxins, but to ascertain that they are in fact the toxins quantified. This is done at considerable cost in sensitivity. For example, the limits of detection for T-2 toxin, diacetoxyscirpenol, HT-2 toxin and zearalenone, are *ca.* 20, 5, 5 and 1 ppb, respectively, using the procedures stated but with only single ion detection.

Selected ion monitoring is a technique which allows for confirmation of materials without resorting to full-scale mass spectrometry, a technique that requires more material. Millard[14] has calculated that it should be possible "to distinguish the compound of interest from perhaps a million compounds chosen at random from all know organic compounds" provided that three ions above *m/z* 350 are monitored. Although some of the ions (*m/z* 290 and 347) we monitored were below *m/z* 350, the selectivity provided by the extraction procedures, normal- and reversed-phase chromatography and the spe-

cific gas chromatography retention times (against internal standards) gave additional specificity. Magnetic sector instruments, such as the one used in this study, are incapable of performing selected ion monitoring for a wide range of ions by changes in accelerating voltage alone because accelerating voltage jumps greater than 10-20% result in defocusing of the ion source with consequent loss of sensitivity. Selected ion monitoring needed for confirmation of the *Fusarium* mycotoxins requires three magnet jumps in addition to the accelerating voltage jumps. Magnet jumps require about a second as compared to accelerating voltage jumps which require milliseconds. This longer jump time results in a sensitivity loss of *ca.* 90% under our conditions. The problem can, for the most part, be alleviated by performing these analyses with a quadrapole mass spectrometer or one of the state-of-the-art magnetic instruments which have faster magnet jump speeds, should even greater sensitivity be needed.

We believe that the described method may be extended to include analysis of deoxynivalenol and possibly nivalenol. Deoxynivalenol is eluted from the reversed-phase Sep-Pak by methanol–water (1:4)[12], and is thus separated from the four mycotoxins we analyzed. Nivalenol, being similar in polarity to deoxynivalenol, most probably has similar elution properties. One could then use the clean-up method published by Scott *et al.*[7] followed by selected ion monitoring as described by Cohen and Lapointe[10] to determine deoxynivalenol and nivalenol.

Internal standards are employed in our method for several reasons. They cancel any variations in quantification caused by instability of instrument electronics[15], allow for greater certainty in assignment of retention times, and increase sensitivity because of the carrier effect[16]. The deuterated analogues of derivatized HT-2 toxin and zearalenone cannot be added before confirmation because they preclude three-ion confirmation of the mycotoxins. For example, loss of $C^2H_3 \cdot$ from [($^2H_{18}$]trimethylsilyl zearalenone gives a peak at *m/z* 462, an ion of the same nominal mass as the molecular ion of trimethylsilyl zearalenone. Furthermore, the deutero-trimethylsilyl zearalenone peaks at *m/z* 445, 436, 350 and 290 interfere with both confirmation and quantification of T-2 toxin because of peak overlap.

A positive feature of our method is speed of analysis. The use of Sep-Paks for clean-up makes it unnecessary to remove pigments with ferric gel[17] (with subsequent emulsion problems) and also makes it unnecessary to spend time performing column or preparative thin-layer chromatography[8]. The specificity and sensitivity of selected ion monitoring provide definite advantages over other electronic methods of detection.

It is important to point out that whenever analyses are carried out at the ppb level in complex matrices such as food, different interferences may occur in different samples. For example, some corn samples gave an *m/z* 290 peak at a retention time close to that of diacetoxyscirpenol. We were able to overcome this problem by monitoring another ion (*m/z* 379). This demonstrates the flexibility available to the analyst in cases where interferences occur and other ions are available for monitoring.

ACKNOWLEDGEMENTS

This research was supported by funds from the State of New Jersey, the U.S. Department of Agriculture (NE-83) and the Charles and Johanna Busch Memorial Fund. This is New Jersey Agricultural Experiment Station Publication No. D-10201-3-83.

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