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Short communication

Simultaneous determination of trichothecene mycotoxins and zearalenone in cereals by gas chromatography–mass spectrometry

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Abstract

Trichothecene mycotoxins are commonly distributed in crop fields in the world, and zearalenone has been found in cereals. Rapid and accurate methods for the determination of these mycotoxins is required to prevent the intoxication of humans, and to contribute to the supply of safe foods and feeds for human and livestock. Gas chromatography–mass spectrometry (GC–MS) is a useful method for the determination of these toxins. We describe here our current GC–MS analysis for the simultaneous determination of trichothecenes and zearalenone contaminants in cereals.

Keywords: Food analysis; Wheat; Trichothecenes; Mycotoxins; Zearalenone

1. Introduction

Trichothecene mycotoxins, such as deoxynivalenol (DON), nivalenol (NIV), and their acetyl derivatives, belong to the secondary toxic metabolites produced by various filamentous fungi, such as *Fusarium*, *Myrothecium*, *Stachybotrys* and *Trichothecium*. These toxins are cytotoxic and inhibit macromolecular synthesis [1,2]. DON and NIV contamination has been found worldwide in cereals, and consumption of these has caused several outbreaks of intoxication in human and animal populations [3–9]. Recently DON, NIV and T-2 toxin (T-2) were found to induce apoptosis in HL-60 cells [10].

Zearalenone (ZEN) is an estrogenic metabolite produced by *Fusarium* species such as *F. culmorum*, *F. crookwellense* (=*F. cerealis*) and *F. graminearum*, and causes hyperestrogenism in livestock [11,12]. ZEN is often found in nature as a co-occurrence of trichothecenes [3–6,9]. Thus, the accurate determination of the cereals contaminated with these toxins is an urgent problem for the supply of safe foods.

Several analytical methods have been carried out for the determination of trichothecenes and ZEN in cereals. Thin-layer chromatography (TLC), gas chromatography (GC), and high-performance liquid chromatography (HPLC) are common techniques for the detection of these mycotoxins for both official and laboratory uses [13–19]. Recently, capillary gas chromatography with (^{63}Ni) electron-capture detection has been performed. It has an excellent sensitivity and can detect 8-keto-trichothecenes at ppb levels; however, it has a disadvantage for the confirmation

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of these mycotoxins. To solve this problem, gas chromatography–mass spectrometry (GC–MS) is introduced for the confirmation. Recent reports have demonstrated that GC–MS is applicable for an accurate analysis of trichothecenes and zearalenone in cereals, foods, and feeds in routine work [9,20–22]. In this paper, we describe the simultaneous determination of seven trichothecenes [DON, 3-acetyl-DON (3ADON), NIV, fusarenon-X (F-X), T-2, neosolaniol (NS), diacetoxyscirpenol (DAS)] and ZEN by using GC–MS.

2. Experimental

2.1. Apparatus and chromatographic conditions

The GC–MS system was composed of a gas chromatograph (HP-5890 series II, Hewlett-Packard, Palo Alto, CA, USA) fitted with a fused-silica column (DB-5, cross-linked 5% phenyl–95% methylsiloxiane, 30 m×0.25 mm I.D., 0.25-μm film thickness, J&W Scientific, CA, USA), an automated sampler (HP-7673), a splitless injector, and a mass spectrometric detector (HP-5971). Column temperature was initially 120°C for 2 min, and increased from 120 to 270°C at a rate of 5°C/min, then raised up to 290°C at a rate of 10°C/min, and held for 8 min. Helium was used as a carrier gas at a flow-rate of 1.1 ml/min (40 cm/s, 13.6 p.s.i. at 120°C; 1 p.s.i.=6894.76 Pa). Injection temperature was 270°C. The mass spectrometer were worked at electron impact at 70 eV, ion source temperature at 180°C, and interface temperature at 280°C.

2.2. Chemicals and reagents

The standards, NIV, DON, 3ADON, FX, T-2, DAS, NS, and ZEN were purchased from Wako (Osaka, Japan). Trimethylsilylating reagents (*N*-trimethylsilylimidazole, trimethylchlorosilane) were purchased from Gasukuro Kogyo (Tokyo, Japan). A Florisil column was used Sep-Pak plus Florisil cartridge column (910 mg) purchased from Waters (Milford, MA, USA). All organic solvents used were of analytical reagent grade.

2.3. Preparation of standards solution

Each stock standard solution of NIV, DON, 3ADON, FX, T-2, DAS, NS and ZEN was dissolved in either methanol or chloroform at concentration of 100 μg/ml and transferred to a 50-ml glass bottle with a screw-cap stopper. Then they were tightly sealed, and stored at 4°C until use. For preparation of mixed working standard solution, an appropriate amount (0.02–0.5 ml) of individual stock standard was transferred to a 50-ml volumetric flask and diluted with chloroform.

2.4. Preparation of samples

A 10-g amount of fine ground sample was poured into a 100-ml Erlenmeyer flask. After adding 50 ml acetonitrile–water (3:1), the flask was shaken with an automatic shaker for 30 min. A 25-ml portion of the solution was filtered through a filter paper (Whatman No. 1), transferred to a 100-ml separation funnel containing 10 ml *n*-hexane, and then defatted with shaking for 10 s. The lower acetonitrile–water layer was transferred to another 100-ml separation funnel, and 1 g of sodium chloride added, then well mixed to separate the two layers. The upper acetonitrile layer was transferred to a 50-ml round-bottomed flask, and evaporated to dryness at 45°C under reduced pressure.

The residue in the round-bottomed flask was dissolved in 2 ml methanol with sonication, and transferred to a 50-ml centrifuge tube. It was further rinsed with 18 ml of chloroform and poured into a centrifuge tube. After centrifugation at 3000 rpm for 5 min, 10 ml of the supernatant were purified by a Florisil column which had been preconditioned with 10 ml chloroform.

After loading to a Florisil column, 20 ml chloroform–methanol (9:1) was added to it for elution of the toxins. The flow-rate was adjusted to about 2 ml/min by weak negative pressure. All of the eluate (30 ml) was collected in a 50-ml round-bottomed flask and the solvent was evaporated using a rotary evaporator at 45°C under reduced pressure. The extract was dissolved in 2 ml methanol with sonication and transferred to a 5-ml PTFE-lined screw-capped vial. The flask was rinsed with a few volumes of methanol. This was transferred into the

Table 1
Retention time and ions monitored for *Fusarium* mycotoxin determination by GC–MS-SIM

Mycotoxin	Retention time (min)	Fragment ions monitored (*m*/*z*)
Deoxynivalenol	27.95	422, 512
3-Acetyldeoxynivalenol	29.66	377, 392
Diacetoxyscirpenol	29.86	350, 378
Fusarenon-X	29.95	480
Nivalenol	30.51	379, 510
Neosolaniol	32.23	350, 436
T-2 toxin	35.60	350, 436
Zearalenone	36.39	462, 350

vial. The methanol solution was heated at 40°C on an aluminum block, and evaporated to dryness under a gentle nitrogen flow. The final purified dry sample was stored in a desiccator until GC–MS analysis.

2.5. GC–MS analysis

To prepare trimethylsilyl (TMS) derivatives of *Fusarium* mycotoxins, the purified sample was dissolved in 0.2 ml of TMS reagent containing *N*-trimethylsilylimidazole–trimethylchlorosilane–ethyl acetate (1:0.2:9), mixed vigorously on a Vortex mixer, and reacted for 15 min at room temperature. Then 0.3 ml *n*-hexane was added to the vial. A 2-μl volume of the TMS derivatives was injected into the GC–MS system.

All spectra were monitored with a total ion current (TIC) and selected ion monitoring (SIM) modes. In the SIM mode, the spectrum of each mycotoxin was analyzed with at least one specific molecular ion selected for the target. The retention time and the selected-ion are given in Table 1.

3. Results and discussion

3.1. Extraction and clean-up procedures

The recovery of DON and especially NIV was improved by using acetonitrile–water (3:1) as the extraction solvent, as previously reported [13]. GC–ECD analysis for the type B trichothecenes (DON, NIV, 3ADON, FX) and HPLC with fluorescence detection for ZEN gave highly sensitive detection; however, in the case of the type A trichothecenes (T-2, DAS, NS) the sensitivities were poor as compared with the type B trichothecenes.

In this experiment, seven trichothecenes and ZEN were simultaneously detected with the same analytical procedure using GC–MS, without special treatments. To save the analytical time and avoid excessive volume of waste solvents, a small-scale protocol was applied for sample preparation using a commercial cartridge column at the clean-up step.

Mycotoxin profiles eluted from a Florisil column are shown in Table 2. The data show that a 25-ml volume of chloroform–methanol (9:1) was sufficient for complete elution of four mycotoxins, including NIV, a trichothecene with higher polarity, from the column. Hence, after 10 ml extracts were loaded to column, 20 ml chloroform–methanol (9:1) were employed for complete elution of the toxins in clean-up procedure.

3.2. Detection and confirmation of trichothecenes and ZEN by GC–MS

GC and GC–MS analyses for trichothecenes and ZEN require to form TMS, trifluoroacetyl (TFA), or heptafluorobutyl (HFB) derivatives [13,23,24]. TFA and HFB derivatives require heat treatment for their

Table 2
Elution profiles of mycotoxins from a Florisil column in a chloroform–methanol system

Mycotoxin	Recovery (%)[a]			Total
Chloroform–methanol (9:1)	0–10 ml	10–20 ml	20–25 ml	
Nivalenol	45.2	49.6	4.3	99.1
Deoxynivalenol	79.2	20.1	0	99.3
T-2 toxin	85.4	13.7	0	99.1
Zearalenone	87.5	11.9	0	99.4

[a] Numbers are mean of duplicate determinations.

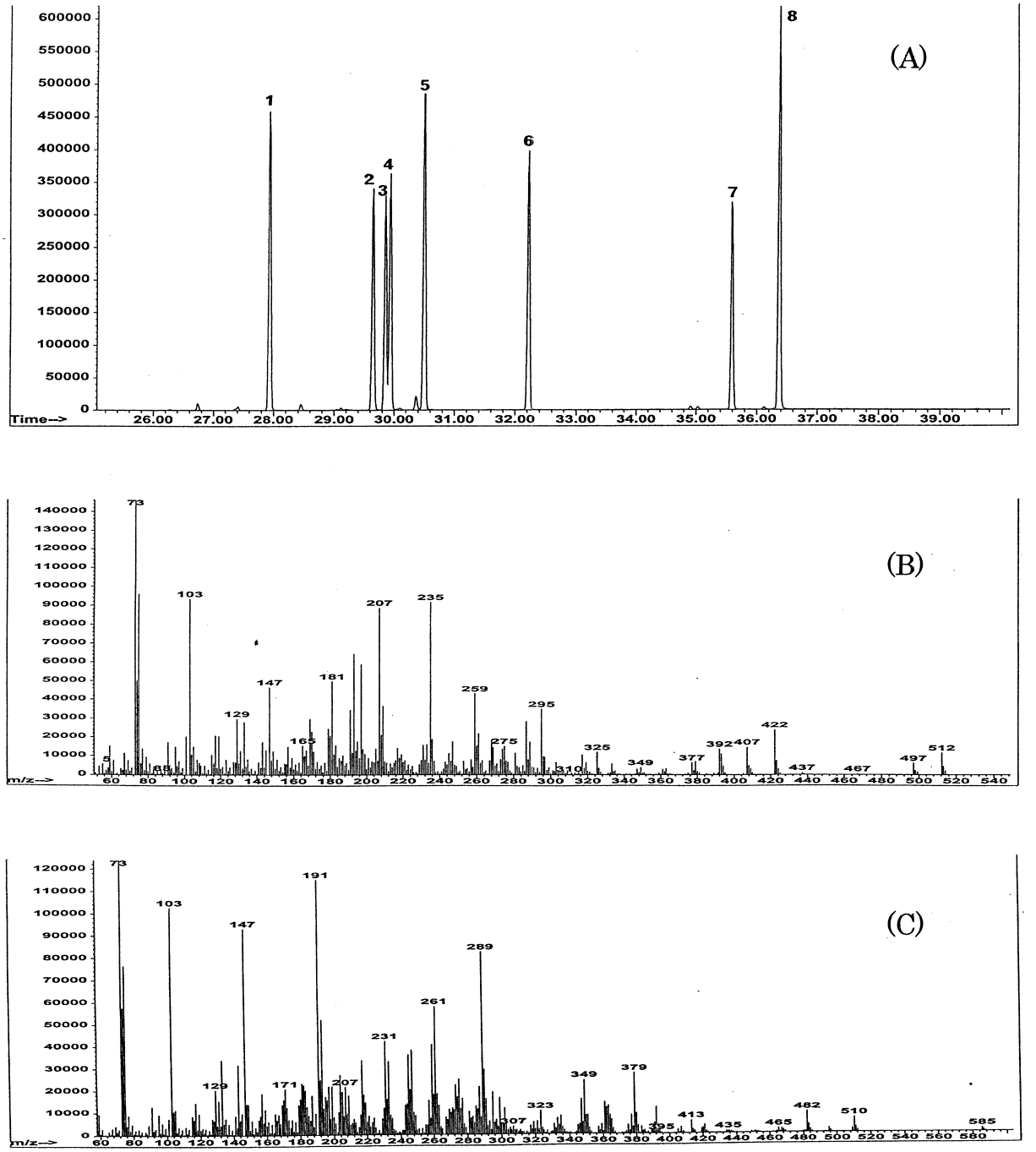

Fig. 1. Total ion chromatogram (A) of TMS derivatives of trichothecenes and zearalenone standard, and mass spectra of deoxynivalenol (B) and nivalenol (C). (1) deoxynivalenol (0.5 ng); (2) 3-acetyldeoxynivalenol (0.5 ng); (3) diacetoxyscirpenol (0.5 ng); (4) fusarenon-X (0.5 ng); (5) nivalenol (0.5 ng); (6) neosolaniol (1 ng); (7) T-2 toxin (1 ng); (8) zearalenone (1 ng).

reactions, and enlarge a molecular formula. Due to a limited maximum mass molecule (about 600–700) of a bench-top type GC–MS, TMS derivatives were used in the present procedure. It took 15 min to form TMS derivatives at room temperature.

A total ion chromatogram of the authentic standards for trichothecenes and ZEN is shown in Fig. 1. Fig. 2 shows the SIM chromatograms of wheat sample contaminated with 34 ng/g of DON and 87 ng/g of NIV. Fragment ions monitored were *m/z* 422, 512 for DON and 379, 510 for NIV. No detectable interfering peaks were observed in SIM chromatograms.

In this method, the detection limits of *Fusarium* mycotoxins in cereals were 5–10 ng/g (DON and

Table 3
Recovery of *Fusarium* mycotoxins from spiked wheat and corn

Mycotoxin	Level spiked (ng/g)	Recovery (%)±RSD (%)[a]	
		Wheat	Corn
Deoxynivalenol	50	86±4.7	84±4.8
3-Acetyldeoxynivalenol	50	88±3.9	85±5.3
Nivalenol	50	83±4.6	81±5.7
Fusarenon-X	50	87±5.0	85±3.6
Diacetoxyscirpenol	50	92±4.3	91±4.4
Neosolaniol	50	93±4.9	89±5.0
T-2 toxin	50	93±4.3	92±4.9
Zearalenone	50	94±5.5	93±3.9

[a] Numbers are mean of triplicate determinations.

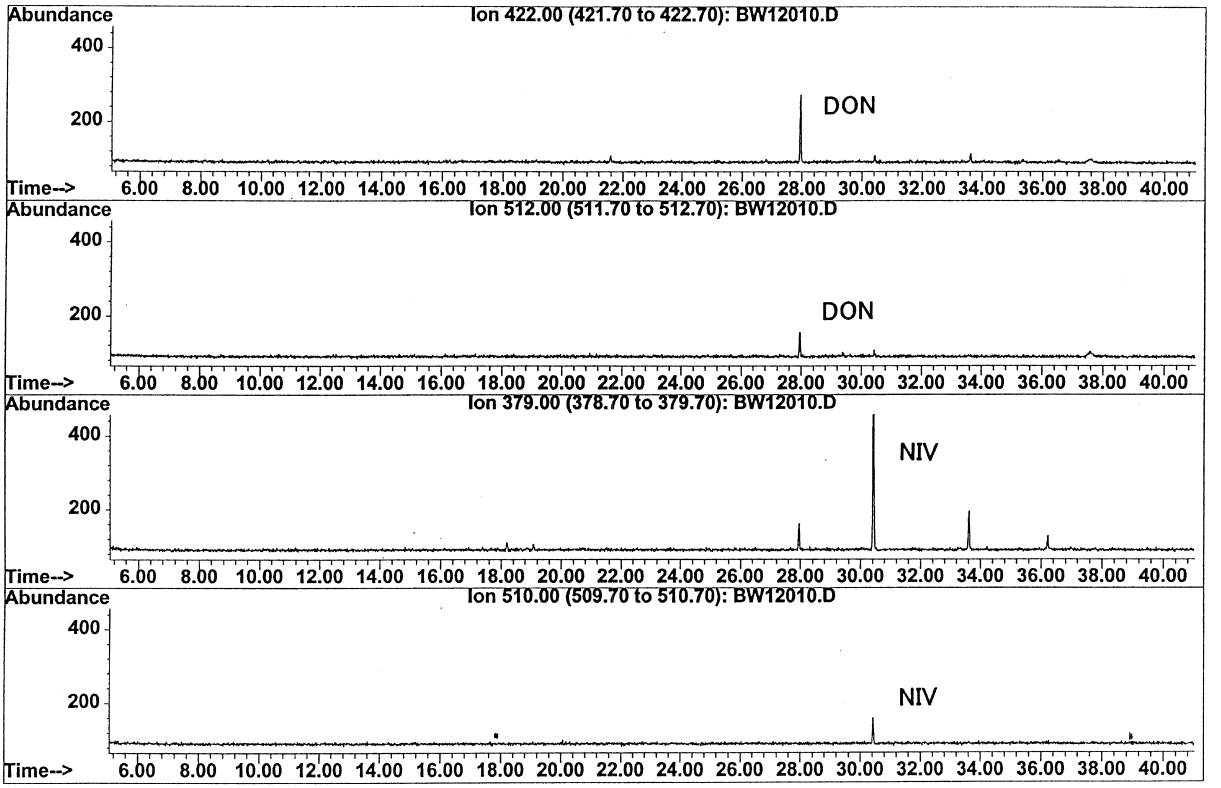

Fig. 2. SIM chromatograms of TMS derivatives of *Fusarium* mycotoxins extracted from wheat, showing contamination with 34 ng/g of deoxynivalenol (DON) and 87 ng/g of nivalenol (NIV). Time scale in minutes.

NIV, 10 ng/g; the others, 5 ng/g) on the basis of three times the average signal-to-noise ratio of the baseline under GC–MS-SIM conditions.

3.3. Recovery of trichothecenes and ZEN from cereals

As summarized in Table 3, the present method showed that eight mycotoxins were recovered from wheat and corn spiked at 50 ng/g concentration, and an average recovery was counted to more than 81%. In additional experiments, the recoveries of mycotoxins from other cereals such as polished rice and barley were more than 84% (data not shown).

4. Conclusion

By adopting the present revised method, the total of organic solvents employed and analytical time required is reduced to less than one-fourth, as compared with those in our previous method. In addition, the GC–MS analysis presented here was adopted for simultaneous detection of eight different *Fusarium* mycotoxins (seven trichothecenes and ZEN) at ppb levels.

We conclude that the GC–MS system described here is a powerful and practical tool for the simultaneous detection and confirmation of trichothecenes and ZEN contaminated in cereals.

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