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Journal of Chromatography A, 815 (1998) 59–65

JOURNAL OF CHROMATOGRAPHY A

Short communication

Direct analysis of several *Fusarium* mycotoxins in cereals by capillary gas chromatography–mass spectrometry

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Abstract

A method for qualitative and quantitative analysis of *Fusarium* mycotoxins by gas chromatography–mass spectrometry (GC–MS) using cold on-column injection was improved. Eight typical mycotoxins, including deoxynivalenol (DON), 3-acetyldeoxynivalenol (3ADN), fusarenon-X (FX), diacetoxyscirpenol (DAS), 15-monoacetylscirpenol (15MAS), T-2 toxin (T-2), scirpentriol (SCT), and zearalenone (ZEA) were subjected to GC–MS without chemical derivatization by means of the on-column injection technique. Chromatographic separation of the toxins extracted from barley was achieved as a single peak, and the specific EI mass spectra of each toxin were obtained. The fatty acids in the extract that interfere with measurements of the toxins on the gas chromatogram were removed by precipitation as an insoluble metal soap with zinc acetate. Additional clean-up was accomplished using a Bond Elut Florisil cartridge. The quantitative detection limit in barley ranged from 0.1 to 0.5 μg/g. The average recoveries of 93.1% for DON, 3ADN, 15MAS, DAS, T-2 and ZEA, and 46.0% for FX and SCT added to barley at the level of 1 μg/g were obtained.

Keywords: *Fusarium* spp.; Food analysis; Mycotoxins; Toxins; Injection methods

1. Introduction

Trichothecene mycotoxins, such as nivalenol (NIV) and deoxynivalenol (DON) and the estrogenic mycotoxin, zearalenone (ZEN) are produced naturally by the fungus *Fusarium* species. Contamination of cereals and feeds with these mycotoxins are known to be associated with several diseases in humans and animals [1]. Several chromatographic methods have been used for qualitative and quantitative analysis of *Fusarium* mycotoxins, including thin-layer chromatography (TLC) [2,3], high-performance liquid chromatography (HPLC) [4] and gas chromatography (GC) [2–7]. Especially, GC has been used for determination of *Fusarium* mycotoxins in cereals. Although direct analysis of some *Fusarium* mycotoxins by capillary GC with cold on-column injection has been well known [8], most analytical methods by GC have employed various chemical derivatizations [2–7]. These chromatographic methods are often unreliable and produce conflicting results, because many endogenous components in cereal samples interfere with mycotoxin analysis. Even GC–mass spectrometry (MS) after chemical derivatization may not be sufficiently specific because of a lack of structural information. One approach to solving this problem is to develop a direct analytical method to measure the native mass spectra of mycotoxins extracted from cereal samples.

The present study was undertaken to analyze

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0021-9673/98/$19.00
PII: S0021-9673(98)00357-4

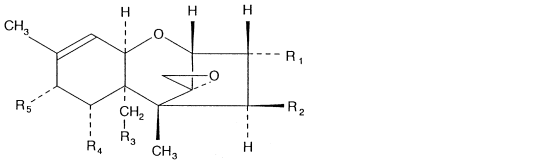
Fusarium mycotoxins by GC–MS without chemical derivatization using an improved cold on-column injection equipped with a laboratory-made inlet liner, keeping the analytical column clean. The method is suitable for the simultaneous detection of native EI mass spectra of *Fusarium* mycotoxins.

2. Experimental

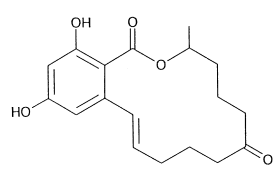
2.1. Mycotoxin standards and reagents

DON, 3-acetyldeoxynivalenol (3ADN), fusarenon-X (FX), diacetoxyscirpenol (DAS), T-2 toxin (T-2), and ZEA with 95% purity were purchased from Wako (Osaka, Japan). Scirpentriol (SCT) and 15-monoacetylscirpenol (15MAS) were kindly provided by Dr. T. Tanaka (Public Health Institute of Kobe City, Kobe, Japan). These mycotoxins were dissolved in chloroform or chloroform–methanol (1:1, v/v) and diluted suitably with acetone prior to use. The chemical structures of the eight mycotoxins are shown in Fig. 1.

All organic solvents used were of pesticide grade (Wako). Zinc acetate and ammonium sulfate were of analytical grade (Wako). Bond Elut FL cartridges (Florisil 500 mg/3 ml) were obtained from Varian (Harbor City, CA, USA) and rinsed with 3 ml of hexane just before use.



toxin	R_1	R_2	R_3	R_4	R_5
deoxynivalenol (DON)	OH	H	OH	OH	=O
3-acetyldeoxynivalenol (3ADN)	OAc	H	OH	OH	=O
fusarenon-X (FX)	OH	OAc	OH	OH	=O
nivalenol (NIV)	OH	OH	OH	OH	=O
scirpentriol (SCT)	OH	OH	OH	H	H
15-monoacetylscirpenol (15MAS)	OH	OH	OAc	H	H
diacetoxyscirpenol (DAS)	OH	OAc	OAc	H	H
T-2 toxin (T-2)	OH	OAc	OAc	H	$OCOCH_2CH(CH_3)_2$



zearalenone(ZEA)

Fig. 1. Chemical structures of *Fusarium* mycotoxins examined in this study.

2.2. Apparatus and operation

A gas chromatograph GC 5890 [Hewlett–Packard (HP), Avondale, PA, USA] equipped with a pressure programmable on-column inlet and a mass spectrometer HP5971 MSD were used. A capillary column (30 m×0.25 μm I.D.) with Rtx-200 bonded stationary phase film of 0.25 μm thickness (Restek, Bellefonte, PA, USA) was used. A glass capillary tube was cut off to make a short glass tube (length 37 mm, 2 mm outside diameter), which provides an inlet liner with a 30-μl volume connected to the capillary column (Fig. 2). Inlet temperature was maintained at 90°C for 0.2 min, programmed from 90–250°C at 100°C/min. Column oven temperature was held at 90°C for 0.2 min, programmed from 90–210°C at 30°C/min and from 210–300°C at 6°C/min. Helium carrier gas was used at a constant flow-rate of 0.75 ml/min. Sample injection was done using an HP 7673 autosampler. The mass conditions were as follows: full scan mode; ionization energy, 70 eV; ion source temperature, 180°C. For quantitative analysis of toxins, selected-ion monitoring was used.

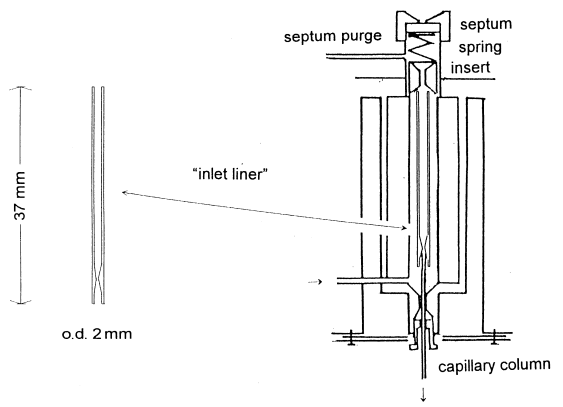


Fig. 2. Design of laboratory-made inlet liner for improved cold on-column injection.

2.3. Extraction and purification of mycotoxins for GC–MS

Barley samples were finely ground and mixed well. Fifteen g of barley powder was swollen with 30 ml of distilled water and allowed to stand for 2 h at room temperature. The swollen barley was extracted with 60 ml of acetonitrile and then filtered by suction. A 30 ml volume of filtrate was mixed with 30 ml of 10% zinc acetate and allowed to stand for 15 min at room temperature. After addition of 10 g of Celite 545, the mixture was filtered again and the filtrate was transferred to a separatory funnel. Three grams of ammonium sulfate were added to the separatory funnel and the mixture was shaken for 5 min. The aqueous layer was re-extracted with 30 ml of acetonitrile. The combined acetonitrile layer was evaporated to dryness. The residue was dissolved in 20 ml of ethyl acetate and centrifuged at 3000 rpm for 10 min at room temperature. The supernatant was evaporated to dryness and then the residue was redissolved in 2.5 ml of chloroform–hexane (1:4, v/v). The sample solution was applied to a Bond Elut FL cartridge, washed with 6 ml of chloroform–hexane (1:4, v/v), and then eluted with 3 ml of chloroform–methanol (4:1, v/v). The eluate was evaporated to dryness under a stream of nitrogen gas in a water bath (40°C). The residue was finally dissolved in 3 ml of acetone, and 2 μl of the acetone solution were injected onto GC–MS.

3. Results and discussion

When toxin standards, DON, 3ADN, FX, DAS, 15MAS, T-2, SCT and ZEA diluted with acetone were injected into the GC–MS using an improved cold on-column injection, a sharp peak was detected at retention time 12.32, 13.79, 14.80, 13.98, 13.77, 17.86, 12.90 and 17.64 min, respectively, showing good peak resolution and high sensitivity on each chromatogram in the scan mode (Fig. 3, chromatogram). DON, however, gave several sub-peaks due to degradative components. In the electron impact ionization (EI) mass spectra, the parent ions of DON, 3ADN, FX and ZEA were observed at m/z 296, 338, 354 and 318 respectively, and specific fragmentations for all toxins analyzed were also observed (Fig. 3, mass spectrum). In addition, the observed mass spectra of toxins except for SCT closely agreed with those of Wiley 130 K mass spectral database. Thus the identification of each toxin became more definite by observing a molecular ion and fragment ions and comparing the observed mass spectrum with that of the authentic mass spectrum library. In this study, we have attempted to analyze NIV in the same way, but direct analysis of the toxin could not been accomplished because of degradation into several peaks after injection. Here, we have accomplished the identification by means of on-column injection technique without chemical derivatization. It is considered that GC–MS–MS could afford a accurate identification of *Fusarium* mycotoxins, including NIV, even with chemical derivatization.

Prior to GC–MS analysis, sample clean up techniques for separation of toxins from fats and oil are indispensable for cold on-column injection. The zinc acetate-treatment [9] and Florisil cartridge column chromatography have been adopted. Fatty acids contained abundantly in cereal could be removed by neither liquid–liquid partition nor column chromatography and often interferred with analysis of mycotoxins. Adachi et al. [9] successfully removed the higher fatty acids from polished rice extracts by zinc acetate treatment in analysis of organophosphorous pesticides. In the case of *Fusarium* mycotoxin analysis, zinc acetate was effective in removing higher fatty acids such as palmitic acid, oleic acid and linoleic acid in cereal as shown in Fig. 4.

Cold on-column injection has been employed for micro-analyses in several fields, such as flavour and petrochemistry. After repeated injections, serious problems are often caused by contamination of many nonvolatile components on the top of column. Our improved cold on-column injection system can keep the top of an analytical column clean by connecting with an inlet liner. After repeated injections, regular replacement of a laboratory-made inlet liner by a fresh one could prevent an analytical column from spoiling, so that GC–MS analysis of toxins by cold on-column injection could be easily performed. Furthermore, the dirty inlet liner was able to be cleaned by treating with hot sulfuric acid overnight.

Fig. 5 shows a selected-ion monitoring chromatogram of added toxins to purified barley extract at 0.2

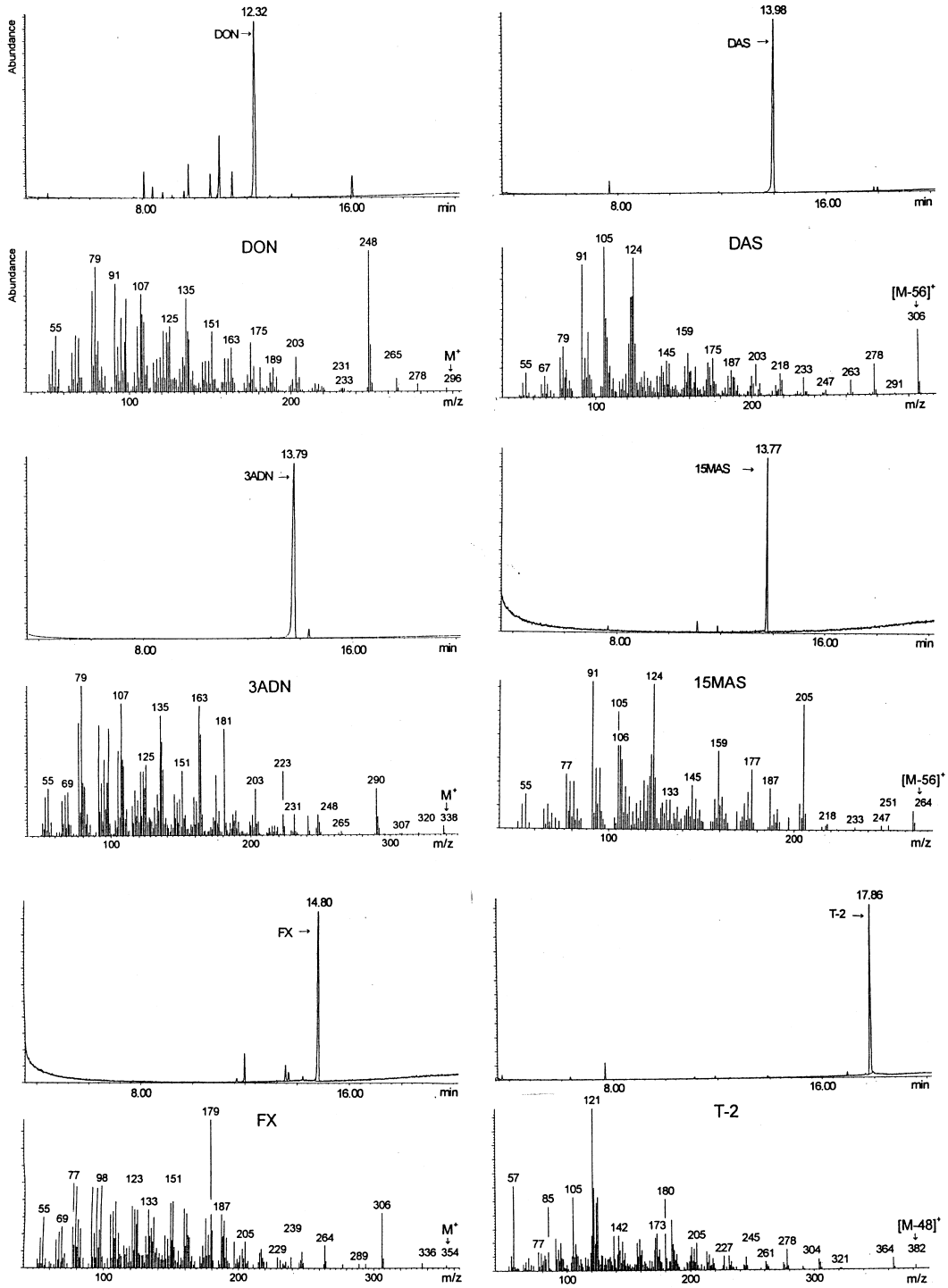


Fig. 3. Gas chromatograms and mass spectra of *Fusarium* mycotoxins. 20 ng each toxin, SCAN mode (m/z 45–450). DON, deoxynivalenol; 3ADN, 3-acetyldeoxynivalenol; FX, fusarenon-X; DAS, diacetoxyscirpenol; 15MAS, 15-monoacetylscirpenol, T-2, T-2 toxin; SCT, scirpentriol; ZEA, zearalenone.

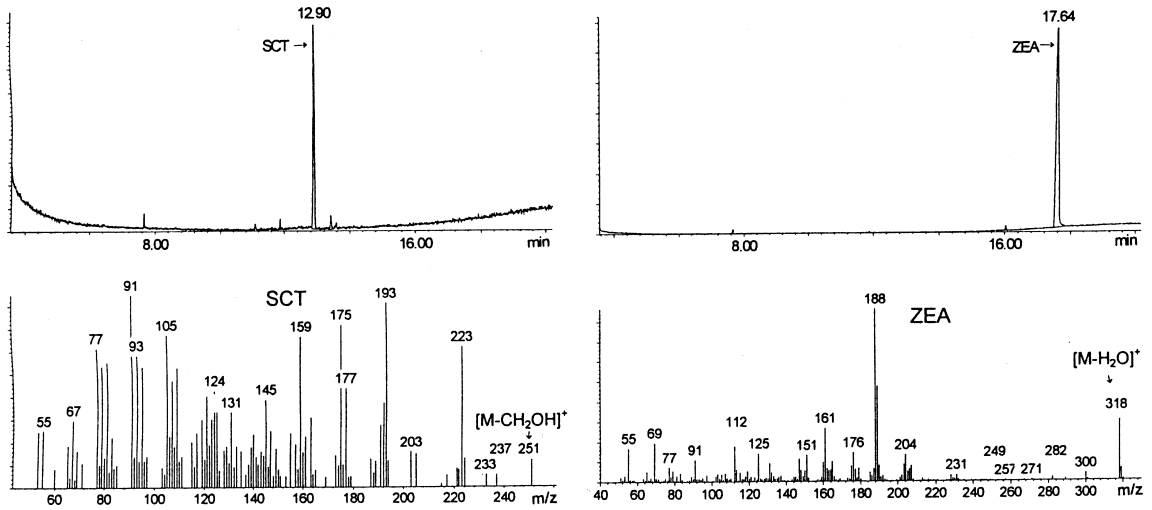


Fig. 3. (*continued*)

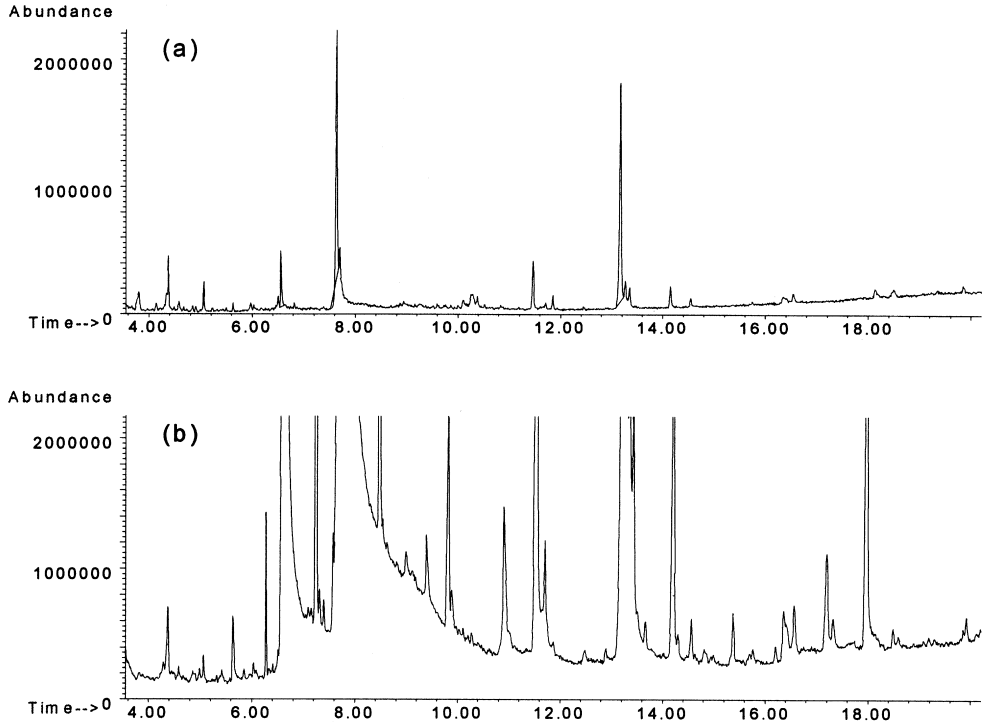


Fig. 4. Total ion chromatograms of barley extract, treated with (a) and without (b) zinc acetate. Analytical conditions are described in Section 2. Time in min.

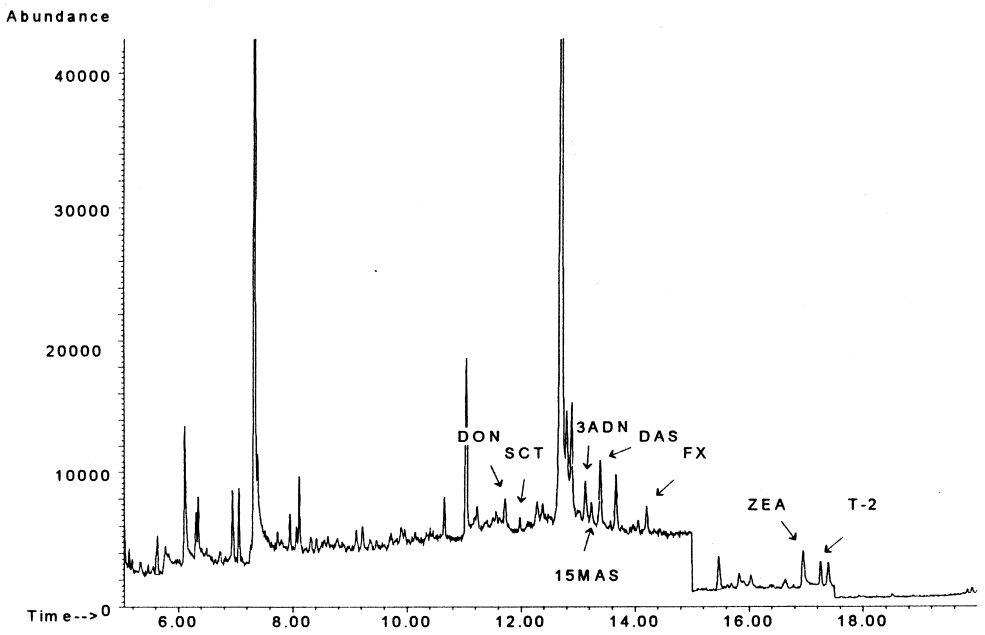


Fig. 5. Selected-ion monitoring chromatogram of barley extract spiked with 0.2 μg/g of each toxin. Monitor ion: DON, m/z=135, 248; SCT, m/z=223, 251; 3 ADN, m/z=223, 290; 15MAS, m/z=205; DAS, m/z=278, 306; FX, m/z=179, 306; ZEA, m/z=188, 318; T-2, m/z=180, 364. Time in min.

μg/ml. The chromatogram reveals that all toxins are successfully separated from sample matrices and detected in barley at relatively low concentration. The detection limit of each toxin in barley extract ranged from 0.1 to 0.5 μg/g by selected-ion monitoring mode.

When eight *Fusarium* toxins at the level of 1 μg were added to 15 g of barley powder, the average recovery for DON, 3ADN, 15MAS, DAS, T-2 and ZEA was 93.1%, however that for FX and SCT was lower, 46.0%, as shown in Table 1. As an additional analysis was tried in the same way followed by chemical derivatization as heptafluorobutyric ester by GC–electron-capture detection (ECD), an average recovery of 85.6% and a relative standard deviation of 15.8% were obtained. It is considered that low recoveries and low reproducibility of toxins are due to slight degradation of these toxins in either GC or MS, because the reproducibility for chemical derivatives as heptafluorobutyric were better than that of native toxins.

Table 1
Recoveries of *Fusarium* mycotoxins[a]

Toxin	Mean (%)	S.D. (%)
Deoxynivalenol (DON)	75.8	22.1
3-Acetyldeoxynivalenol (3ADN)	82.0	28.0
Fusarenon-X (FX)	49.4	29.1
Scirpentriol (SCT)	42.5	29.2
15-Monoacetylscirpenol (15MAS)	97.3	9.4
Diacetoxyscirpenol (DAS)	107.5	8.1
T-2 Toxin (T-2)	98.0	22.4
Zearalenone (ZEA)	98.2	17.9

[a] Toxins in barley added at 1 μg/g (n=4).

Our purification procedure and GC–MS analysis with an improved cold on-column injection system is more adequate than other conventional methods for *Fusarium* mycotoxins from the following points of view; simple, rapid purification without chemical derivatization and the accurate identification of toxins by inspecting their native mass spectra.

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