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Journal of Chromatography A, 858 (1999) 175–185

JOURNAL OF CHROMATOGRAPHY A

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Determination of the *Fusarium* mycotoxins, fusaproliferin and beauvericin by high-performance liquid chromatography–electrospray ionization mass spectrometry

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Received 9 March 1999; received in revised form 16 July 1999; accepted 16 July 1999

Abstract

A method is described using LC–MS for the detection of the mycotoxins fusaproliferin (FUS) and beauvericin (BEA) in cultures of *Fusarium subglutinans* and in naturally contaminated maize. Protonated molecular ion signals for FUS and BEA were observed at m/z 445 and m/z 784, respectively. Collision induced dissociation of the readily dehydrated protonated molecular ion of the sesterterpene FUS (m/z 427) led to the loss of another water molecule (m/z 409) and acetic acid (m/z 385), while the cyclic lactone trimer BEA fragmented to yield the protonated dimer (m/z 523) and monomer (m/z 262), respectively. Detection of FUS was best performed in the MS–MS mode while BEA displayed a stronger signal in the MS mode. The on-column instrumental detection limits for pure FUS and BEA were found to be 2 ng and 20 pg (S/N=2) while those in naturally contaminated maize were 1 μg/kg and 0.5 μg/kg, respectively. Five South African strains of *F. subglutinans* were analyzed following methanol extraction of which four produced FUS at levels between 330 mg/kg and 2630 mg/kg while only three produced BEA at levels between 140 mg/kg and 700 mg/kg. Application of this method to naturally contaminated maize samples from the Transkei region of South Africa showed FUS at levels of 8.8–39.6 μg/kg and BEA at 7.6–238.8 μg/kg. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: *Fusarium subglutinans*; Food analysis; Mycotoxins; Fusaproliferin; Beauvericin

1. Introduction

Fusarium mycotoxins continue to occur in agricultural commodities as a result of fungal contamination hence presenting serious animal and human health problems. Various *Fusarium* species have recently been found to produce the two mycotoxins, fusaproliferin (FUS) and beauvericin (BEA). FUS is a bicyclic sesterterpene (Fig. 1A) derived from five isoprenic units. This compound was first purified from corn kernel cultures of a strain of *Fusarium proliferatum* (Matsushima) Nirenberg isolated from corn ear rot in Northern Italy [1,2]. Subsequent investigations resulted in the detection of this compound in maize kernel cultures of several isolates of *F. proliferatum* and *F. subglutinans* (Wollenw. and Reinking, Toussoun and Marasas) [3] and it was found as a natural contaminant of pre-harvest maize kernels in Italy [4]. FUS has been found to be toxic

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PII: S0021-9673(99)00814-6

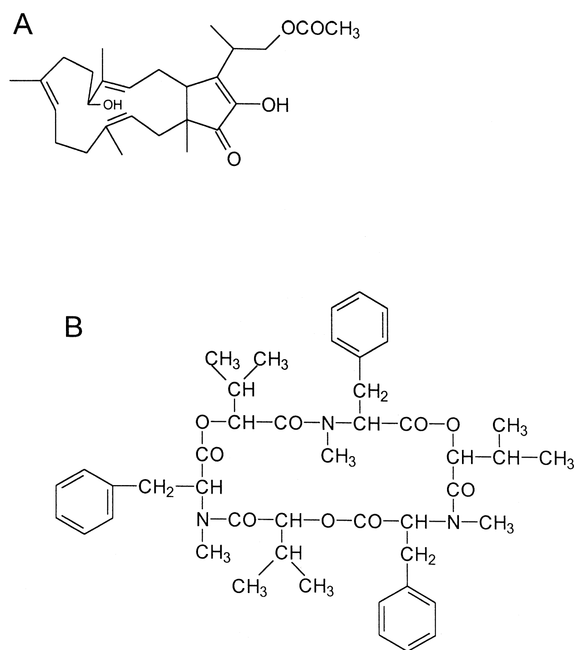

Fig. 1. Chemical structures of (A) fusaproliferin and (B) beauvericin.

to brine shrimps (*Artemia salina* L.), the lepidopteran cell line SF-9 (insect cells) and the IARC/LCL171 human nonneoplastic B-lymphocyte cell line [5] as well as to cause teratogenic effects on chicken embryos [6].

BEA on the other hand is a cyclohexadepsipeptide containing an alternating sequence of three *N*-methyl L-phenylalanyl and three D-α-hydroxyisovaleryl residues (Fig. 1B). This compound, first reported to be produced by some entomopathogenic fungi such as *Beauveria bassiana* (Balsamo) Vuill. [7] and *Paecilomyces fumosoroseus* (Wize) Brown and Smith [8] has also been detected in an entomopathogenic culture of *F. subglutinans* [9]. BEA is structurally similar to the enniatins, which are also produced by a number of *Fusarium* species, and differs from them only in the nature of the *N*-methylamino acid [10]. More recently, several isolates of *F. subglutinans* from maize [11,12] and *F. proliferatum* [13–16] were shown to produce BEA. It was also detected as a natural contaminant up to 60 mg/kg in Polish maize [17] and later in maize from Italy [15,18,19]. BEA is highly toxic to insects [10] and to murine and human cells, in which it induces apoptosis [20–22].

In view of the recognized adverse biological effects caused by these mycotoxins, monitoring their levels in *Fusarium* infected maize samples is important to evaluate the risk due to human and animal consumption of contaminated maize. Current analyses of maize extracts are performed by high-performance liquid chromatography (HPLC) with UV detection at 261 nm for FUS and 225 nm for BEA [19]. A detection limit of 1 mg/kg maize for both toxins has been reported, while another publication by Krska et al. [23] reported on improved detection at 192 nm for BEA as the maximum absorption was observed to occur at this wavelength. This detection wavelength combined with the appropriate sample clean up using solid-phase extraction (SPE) cartridges resulted in a detection limit of 0.05 mg/kg maize.

In order to achieve adequate sensitivity and specificity, an analytical technique must overcome serious matrix interferences, which by UV detection are sometimes difficult to eliminate and may require multiple sample clean up steps resulting in time consuming analyses. Preparation of maize extracts involved extraction with methanol–1% aqueous sodium chloride followed by a defatting step with *n*-hexane and an extraction of the methanol layer with dichloromethane [17]. However, in a subsequent report [24], considerable losses were observed during the defatting step, hence this step was omitted and the dichloromethane fraction applied to a SPE cartridge and BEA eluted with a mixture of chloroform–methanol (98:2, v/v). Mean recoveries of 82% were reported. The work of Josephs et al. [25] subsequently described the use of Mycosep columns followed by SPE clean up on silica columns for the purification of BEA from maize and cereals. This method reported a mean recovery of 96% and enabled a much greater sample throughput than previous methods although no improvement in the detection limit was reported.

Liquid chromatography–mass spectrometry (LC–MS) has over recent years acquired great popularity and many applications of this technique to food analysis have been reported [26]. MS provides high selectivities of detection owing to the ability to

separate or filter ions according to their mass-to-charge ratios (m/z). This selectivity, in addition to the resolution of chromatographic separation, adds great value to this technique. In combination with selected ion monitoring (SIM) or selected reaction monitoring (SRM), highly structure-specific detection of these mycotoxins can be achieved, even with complex matrices, hence eliminating the need for time consuming sample clean ups.

This study reports for the first time the application of LC–MS and LC–MS–MS for the determination of FUS and BEA in cultures of South African isolates of *F. subglutinans* isolated from Transkeian maize. The method was further applied to the analysis of naturally contaminated maize and is the first report of the natural occurrence of these toxins in Transkeian maize.

2. Experimental

2.1. Chemicals and solvents

Pure BEA was purchased from Sigma (St. Louis, MO, USA) while FUS was obtained from Dr. A. Ritieni (University of Naples, Italy). Acetonitrile and methanol (HPLC-grade) were obtained from BDH (Poole, UK) while formic acid (analytical grade) was obtained from Merck (Darmstadt, Germany). Water for HPLC mobile phase was purified in a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Sample details

Five strains of *F. subglutinans* (MRC 115, 1077, 1093, 1084, 1097) previously isolated from maize in the Transkei region of South Africa, were grown in the dark on autoclaved maize in fruit jars at 25°C for three weeks, harvested and dried at 50°C for 12 h. The maize was then ground in a laboratory mill to pass through an 840-μm sieve and was subsequently well mixed.

Four naturally contaminated maize samples were collected from two regions within the Centane district in the Transkei region during 1997. These samples were hand-selected as showing visible *Fusarium* infection.

2.3. Sample extraction

Extracts from the cultures were prepared by homogenizing 20 g of culture material from each strain in 100 ml methanol for 5 min using a Polytron homogenizer (Kinematica, Luzern, Switzerland) [19]. The extracts were centrifuged on a Sorvall RC-3B refrigerated centrifuge (DuPont, CT, USA) at 4°C and at 4000 *g* for 5 min and filtered (Whatman No. 4). A 5-ml volume of the filtrate was placed into a vial and evaporated to dryness at 55°C under a constant flow of nitrogen. No clean up was performed on the samples.

Extracts of naturally contaminated maize were similarly prepared. In brief, 20 g of sample was homogenized in 100 ml methanol and 30 ml removed and evaporated to dryness. The extracts were reconstituted into 1 ml of mobile phase prior to injection. Once again no clean up was performed on the samples.

2.4. HPLC conditions

HPLC analysis was carried out using a SpectraSERIES P2000 pump equipped with an AS 1000 autosampler and a UV 1000 variable-wavelength UV detector (all from Thermo Separation Products, Riviera Beach, FL, USA). The extracts of the cultures and the maize samples were reconstituted in mobile phase, filtered through a 0.45-μm syringe filter (Millipore, Yonezawa, Japan) and injected (20 μl) onto the column. The toxins were separated by binary gradient elution on a 150×4.6 mm I.D. Luna C_{18} reversed-phase column (Phenomenex, Torrance, CA, USA) packed with 5 μm ODS-2. Solvents A and B consisted of water–acetonitrile–formic acid in the ratios 90:10:0.1 and 10:90:0.1, respectively. The mobile phase consisting of A–B (30:70) was pumped at a flow-rate of 0.5 ml/min through the column for 5 min following injection of the sample. The mobile phase composition gradient was thereafter adjusted in a linear profile over 3 min to 100% B which was retained for a further 7 min prior to being adjusted back to A–B (30:70) within 3 min.

UV detection was performed on-line prior to MS detection.

2.5. MS conditions

Positive ion electrospray ionization (ESI) mass spectrometry was performed using a Finnigan MAT LCQ ion trap mass spectrometer (San Jose, CA, USA). MS parameters were optimized separately for FUS and BEA by direct infusion of 50 μg/ml FUS and 25 μg/ml BEA standards at 3 μl/min into the source. During LC–MS, the LC effluent entered the mass spectrometer without splitting at a source voltage of 5.0 kV for FUS and 4.5 kV for BEA. The use of appropriate segments during the chromatographic run made scanning at optimum conditions possible for each of the target analytes. The heated capillary temperature was maintained at 250°C, sheath gas at 80 arbitrary units, auxiliary gas at 10 arbitrary units while the capillary voltage was set at 41 V for FUS and 46 V for BEA, respectively. Initially, the mass spectrometer was programmed to perform full scans between m/z 387–467 and m/z 744–824 for FUS and BEA in order to observe the protonated molecular ion signal of these compounds at m/z 445 and m/z 784, respectively, as well as possible fragment ions and adducts. The final analytical determination of FUS, however, was done in the MS–MS mode through collision induced dissociation (CID) (collision energy 22%) of its readily dehydrated protonated molecular ion at m/z 427. The resulting product ions at m/z 367 and m/z 349 were monitored using an isolation width of 2 amu for each ion. However BEA was determined in the MS mode. Quantitation was achieved by comparing the peak areas of the toxins with the corresponding calibration plot of the standards.

2.6. Detection limits and response profile

A series of standards ranging from 0.1 μg/ml to 5 μg/ml for FUS and 0.001 μg/ml to 1 μg/ml for BEA were injected in order to determine their on-column instrumental detection limits, respectively. The linearity of calibration plots for both toxins were determined over the designated calibration range. The precision of the measurement of FUS and BEA was readily determined by performing triplicate injections of each compound under identical experimental conditions. The extracts were diluted so as to yield FUS and BEA responses within the experimental range of the calibration plots. Analytical recoveries for FUS and BEA were determined following spiking experiments at 15 mg/kg and 4 mg/kg, respectively in duplicate on clean maize.

3. Results and discussion

3.1. MS tuning

The full-scan positive ion ESI mass spectrum for FUS and BEA, obtained by continuous infusion into the source, is shown in Fig. 2. The mass spectrum of FUS showed the protonated molecular ion at m/z 445 and a prominent signal at m/z 427 corresponding to the dehydrated protonated molecule. The mass spectrum of BEA, on the other hand, consisted of the protonated molecular ion at m/z 784 together with ammonium, sodium and potassium adducts at m/z 801, m/z 806 and m/z 822, respectively. Fig. 3 shows the chromatographic separation of 10 ng FUS and BEA. During the chromatographic run, the mass spectrometer was programmed into two segments which allowed for the optimal detection of each toxin. Full scans were performed between m/z 387 and m/z 467 for FUS in segment one and between m/z 744 and m/z 824 for BEA in segment two. FUS was observed to provide an extremely weak signal in comparison to BEA. Unlike conventional detectors such as UV and fluorescence, which respond to intrinsic physical properties of the molecules in solution, electrospray MS detection requires transfer of ions from liquid to vapor phase. This process is known to produce various response levels depending on molecular type. It has been postulated that these differences can be due to solution equilibria between the interior and the surface of the electrospray charged droplet or due to the way the unsolvated ions are formed from the charged droplet [27]. Alternatively, the stability of the ion during the electrospray process may play a role and in this regard it was observed that FUS readily formed the dehydrated ion at m/z 427 which was always more abundant than the protonated molecular at m/z 445

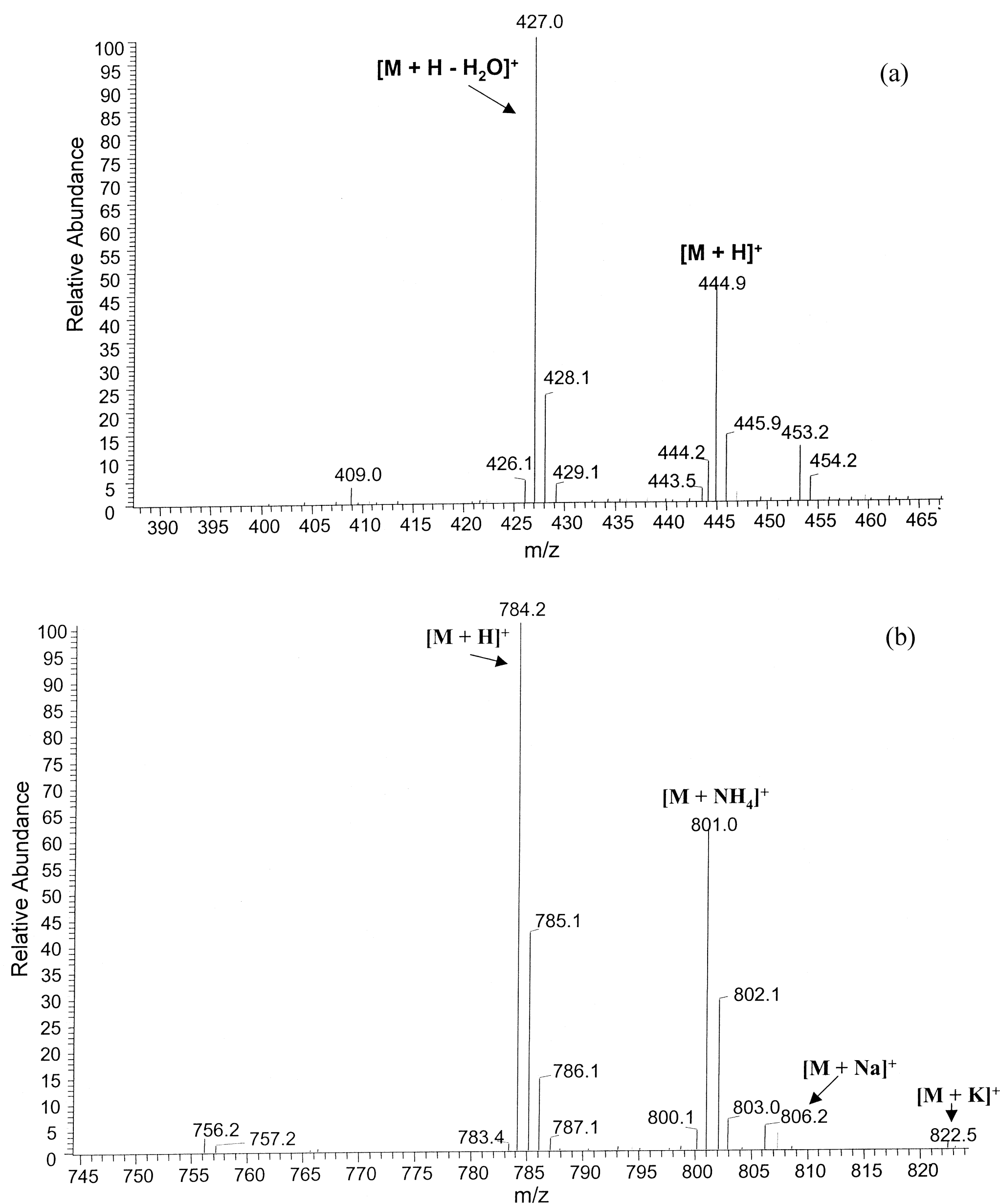

Fig. 2. Full-scan positive ion ESI-mass spectrum of (a) fusaproliferin and (b) beauvericin showing the protonated molecular ion of each toxin. MS experimental conditions are given in Section 2.5.

in the ESI mass spectrum. Furthermore, in the MS mode, a detection window of 80 amu was used which means that if FUS fragmented into ions below m/z 387 then these ions would not have been detected by the mass spectrometer thus leading to a lower response. A larger detection window on the other hand would have resulted in greater levels of background noise.

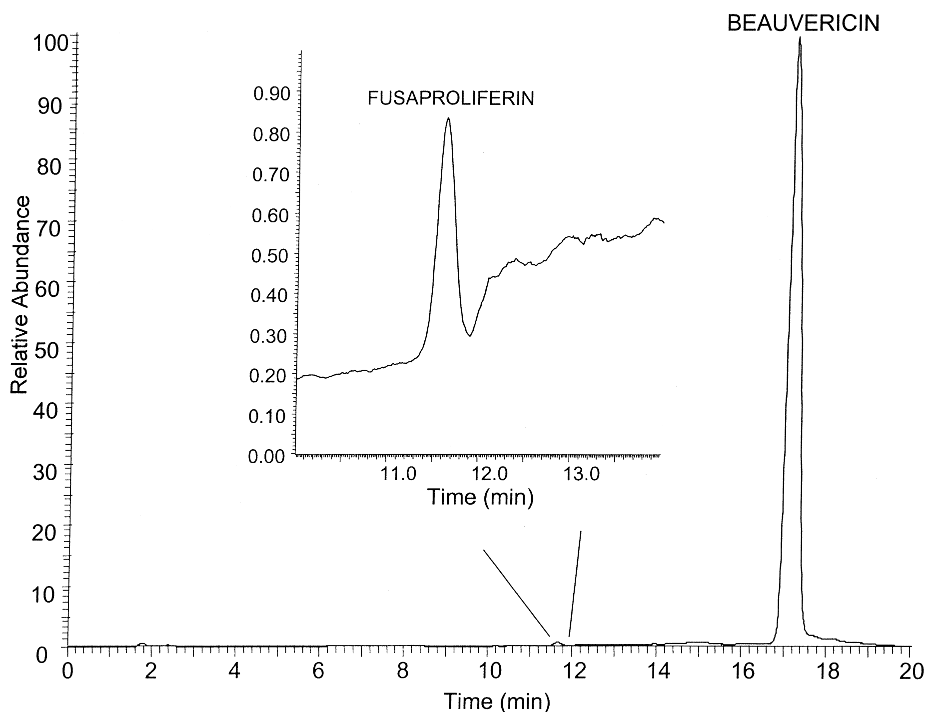

Fig. 3. Total ion chromatogram of fusaproliferin and beauvericin resulting from the injection of 10 ng of each toxin. Separation was performed by gradient elution on a 150×4.6 mm I.D. Luna C_{18} reversed-phase column packed with 5 μm ODS-2. For HPLC gradient conditions, see Section 2.4.

3.2. Collision induced dissociation of FUS and BEA

In addition to molecular mass information obtained by MS, MS–MS and MS–MS–MS experiments were performed through CID to yield structurally specific data. These modes add supplementary mass filters to the standard LC–MS step allowing the characterization of compounds not only by their molecular ions, but also by their specific fragmenta-

Table 1
Fragment ions observed for FUS and BEA under CID MS–MS and MS–MS–MS experiments on the ion trap mass spectrometer

	m/z of fragment ions observed	Interpretation
Fragmentation of FUS		
MS–MS of *m/z* 427	409	$[M+H-2H_2O]^+$
	385	$[M+H-CH_3COOH]^+$
	367	$[M+H-H_2O-CH_3COOH]^+$
	349	$[M+H-2H_2O-CH_3COOH]^+$
MS–MS–MS of *m/z* 367	349	$[M+H-2H_2O-CH_3COOH]^+$
Fragmentation of BEA		
MS–MS of *m/z* 784	541	$[Dimer+H+H_2O]^+$
	523	$[Dimer+H]^+$
	262	$[Monomer+H]^+$
MS–MS–MS of *m/z* 541	523	$[Dimer+H]^+$
	262	$[Monomer+H]^+$

tion. During the time that the ions are resident in the ion trap, it is possible to selectively excite ions of specific mass-to-charge ratios and produce diagnostic fragment ion spectra, which are characteristic of the structural moieties present in the analytes. This two-stage filtering process allows increased specificity of

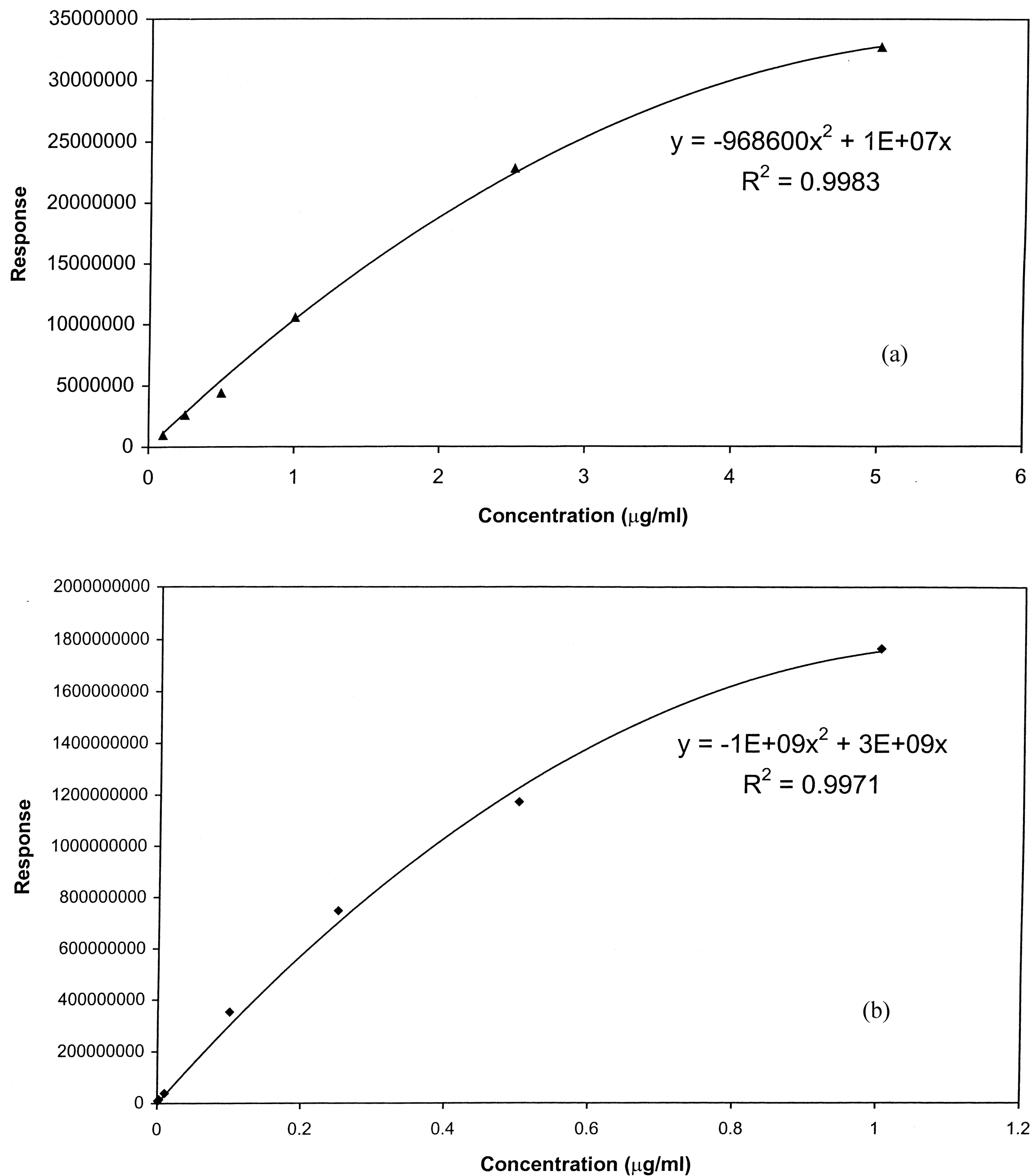

Fig. 4. Calibration plots of (a) fusaproliferin and (b) beauvericin within the experimental concentration range. Responses were obtained following detection in MS–MS and MS modes, respectively.

detection even in the presence of co-eluting contaminants.

In order to study the fragmentation of FUS and BEA, the collision energy was sequentially increased while continuously infusing 25 μg/ml of each toxin separately into the source. The fragmentation of FUS both in MS–MS and MS–MS–MS modes yielded product ions corresponding to the loss of water and acetic acid simply by increasing the collision energy from 15% to 20% (Table 1). BEA, which is a cyclic lactone trimer containing three D-α-hydroxy-isovaleryl-*N*-methyl L-phenylalanyl residues, fragmented to yield characteristic dimer and monomer product ions resulting from the cleavage of the amide bond (Table 1). The collision energy was increased from 15% to 35% and during this process, the molecular ion signal was observed to decrease while the signals corresponding to the protonated dimer and monomer increased. A MS–MS–MS experiment was further performed by selecting the product ion at m/z 541 and subjecting this ion to 20% collision energy upon which resulting fragments at m/z 523 and m/z 262 were once again observed. Although MS–MS–MS data were not used for quantitation, it is nevertheless important for confirmation purposes.

3.3. Detection limits

The detection limit for FUS in the MS–MS mode and BEA in the MS mode was found to be 2 ng and 20 pg, respectively ($S/N=2$) per 20 μl injection. The limit of quantitation, however, was found to be 5 ng and 200 pg ($S/N=10$) for FUS and BEA, respectively. FUS showed a linear response up to 2.5 μg/ml whereas the response to BEA was linear up to 0.25 μg/ml. At higher concentrations, both calibration plots deviated from linearity (Fig. 4a and b). This indicated that in order to obtain linear responses, one had to work at low concentrations. The precision of the measurement for FUS and BEA standard solutions was found to have a RSD of 1.07% and 0.05% at the 0.05 μg/ml level, respectively. The recoveries from spiked maize samples following methanol extraction were 71% and 94% for FUS and BEA, respectively with the overall precision of the method having a RSD of 7%. The limit of detection of FUS and BEA in maize was found to be 1 μg/kg and 0.5 μg/kg, respectively.

3.4. Analysis of strains of *F. subglutinans*

Five South African strains of *F. subglutinans* were cultured on maize kernels and analyzed for the production of the two toxins using both MS and MS–MS modes of analysis and the results are summarized in Table 2. Four of the five strains analyzed produced FUS at levels between 330 mg/kg and 2630 mg/kg while three strains produced BEA at levels between 140 mg/kg and 700 mg/kg. In particular, the strains MRC 1077, MRC 1084 and MRC 1093 produced both toxins. The strain MRC 1097 produced neither FUS nor BEA. Analysis of FUS in the MS mode led to a rising baseline during the chromatographic process, while analysis of the fragment ions following CID eliminated this problem adding greater credibility to the quantitation of FUS (Fig. 5a). Detection of BEA was also performed in the MS–MS mode, however the minimum detectable quantity injected on column was 10 ng indicating that the MS mode produced detection limits 500-times lower than in the MS–MS mode. Furthermore, apart from the differences in detection limits ob-

Table 2
FUS and BEA levels in cultures of five strains of *F. subglutinans* (experiments were performed in the MS–MS and MS modes for FUS and BEA analyses, respectively)

Strains of *F. subglutinans* analyzed	Concentration of FUS (mg/kg)	Concentration of BEA (mg/kg)
MRC 115	1250	ND[a]
MRC 1077	2630	370
MRC 1084	330	700
MRC 1093	540	140
MRC 1097	ND	ND
Control maize	1	ND

[a] ND=Not detected (<5 mg/kg for culture and <0.3 mg/kg for control maize).

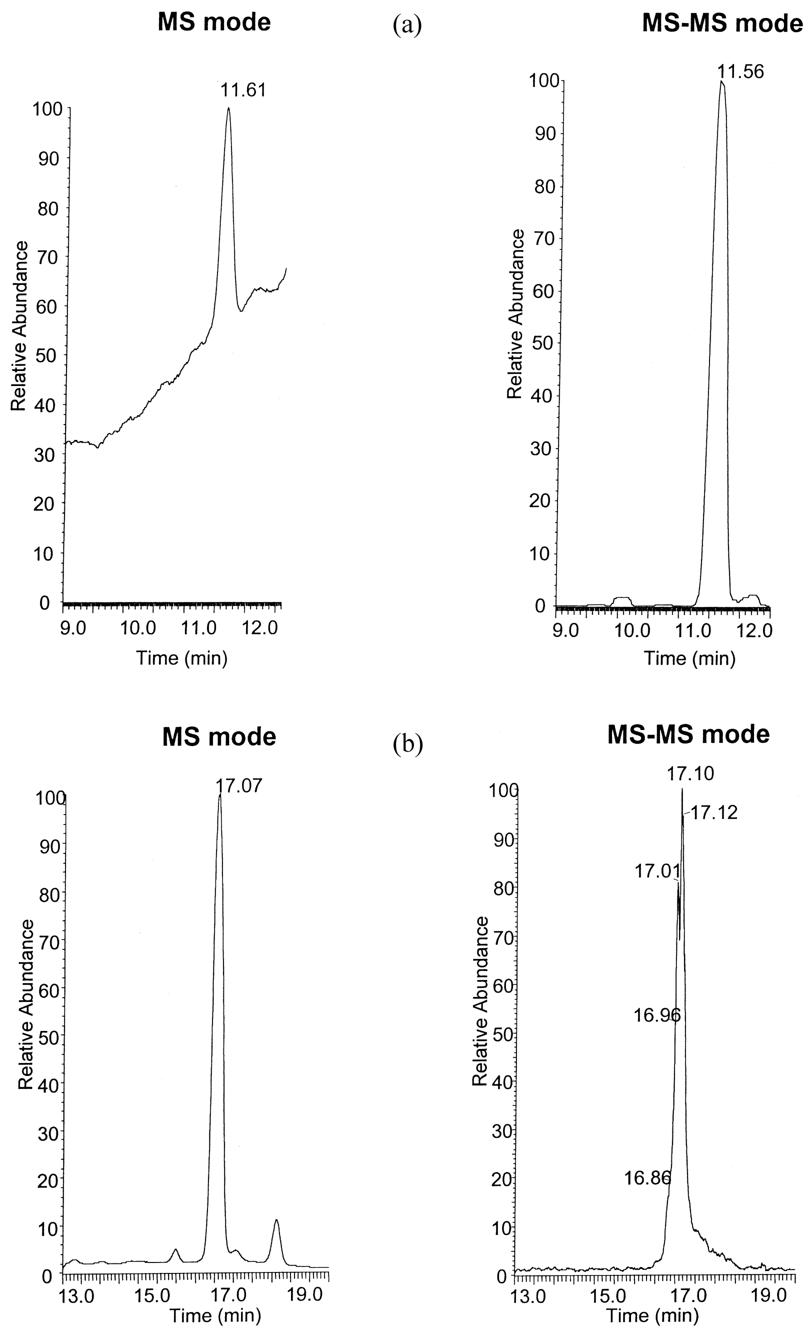

Fig. 5. The MS and MS–MS detector response to (a) fusaproliferin and (b) beauvericin in culture material illustrating the choice of detection mode best suited for each toxin.

Table 3
FUS and BEA levels in four naturally contaminated maize samples obtained from two different regions within the Centane district of Transkei, South Africa (experiments were performed in the MS–MS and MS modes for FUS and BEA analyses, respectively)

Sample number	Concentration of FUS (μg/kg)	Concentration of BEA (μg/kg)
D4B	8.8	7.6
D8B	ND[a]	8.4
F3B	16.8	193.4
F6B	39.6	238.8

[a] ND=Not detected (<1 μg/kg).

tained for BEA using both modes, the peak shape was markedly improved upon specifically monitoring the protonated molecular ion (Fig. 5b).

F. subglutinans is an important pathogen of maize [28,29] and other crops that are used in animal feeds and human foods. Previous investigations of these strains by LC–MS [30] also revealed their ability to produce moniliformin, another toxic secondary metabolite. These results indicate that the toxicity of *F. subglutinans* may not only be dependent on moniliformin, but also due to the presence of FUS and BEA. The co-occurrence of these toxins may have synergistic effects on the overall toxicity of the strains, as a result food and feed contamination by this fungus may be a greater problem than initially anticipated.

3.5. Analysis of naturally contaminated maize

Due to the worldwide distribution of *F. subglutinans* on maize [11,17,28,29] and the ability of the fungus to produce FUS and BEA, these toxins were regarded as potential contaminants of maize. Consequently, four maize samples showing visible *Fusarium* infection were analyzed to demonstrate the ability of the technique to perform the analysis effectively in natural samples. Table 3 summarizes the results obtained for the four maize samples. These results indicate for the first time the natural occurrence of both FUS and BEA in visibly mouldy home-grown maize in the Transkeian region of South Africa. These results also reveal the ability of LC–MS and LC–MS–MS to analyze naturally contaminated samples without the need for sample clean up. A recent publication [31] described the natural occurrence of FUS and BEA in *Fusarium*-contaminated livestock feed in Iowa, USA. This report, supplemented by our current findings, suggests that these toxins may be more widespread than initially thought, hence warranting further investigations into the natural occurrence of these toxins.

4. Conclusion

This study has successfully demonstrated the potential of LC–ESI-MS and LC–ESI-MS–MS in rapidly assessing the FUS and BEA contaminant levels in maize. The high degree of sensitivity and specificity of determination allowed for the detection of low levels of these toxins without sample clean up, thereby eliminating the previously reported analyte losses prior to analysis with HPLC–UV. The ability of the South African strains of *F. subglutinans* to produce these toxins, amongst others, adds concern to the possible synergistic harmful effects caused by consumption of contaminated maize. In culture material, levels of FUS and BEA as high as 2630 mg/kg and 700 mg/kg, respectively, were detected while in naturally contaminated maize samples, levels of FUS and BEA as low as 8.8 μg/kg FUS and 7.6 μg/kg BEA were readily detected.

Acknowledgements

We gratefully acknowledge The Wellcome Trust for financial support towards the purchase of the LC–MS. A.R. acknowledges the International Relationship Office of the University of Naples ‘‘Federico II’’ for financial support towards his visit to PROMEC. The authors also wish to thank Dr. J.P. Rheeder for the Transkeian maize samples and Ms. M. Reeders and Mr. T. Leukes for technical assistance in the production of culture material.

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