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Determination of the mycotoxin moniliformin in cultures of *Fusarium subglutinans* and in naturally contaminated maize by high-performance liquid chromatography–atmospheric pressure chemical ionization mass spectrometry

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Abstract

A LC–MS method employing triethylamine as ion-pairing reagent for the determination of moniliformin in culture material and naturally contaminated maize samples is described. Mass spectrometric detection of moniliformin was accomplished following atmospheric pressure chemical ionization to yield the deprotonated molecular ion $[M-H]^-$ at m/z 97. The moniliformin response was found to be linear over the injected range 10 ng to 700 ng and a detection limit of 10 ng was attainable at a signal-to-noise (S/N) ratio of 4. Five South African strains of *Fusarium subglutinans* were grown on maize kernels and moniliformin extracted with an acetonitrile–water (95:5) mixture. Following sample clean up with reversed-phase (C_{18}) solid-phase extraction cartridges, the extracts were subjected to LC–MS analysis. Triethylamine was used as an ion-pair reagent and found to improve the retention characteristics of moniliformin without any detrimental effects to the instrument. Moniliformin concentrations ranged between 130 mg/kg and 1460 mg/kg culture. Application of this method to naturally contaminated maize samples from Transkei showed that it was capable of measuring moniliformin levels down to 10 μg/kg in selected moldy maize cobs. This is the first report on the application of LC–MS to the analysis of moniliformin in cultures of *F. subglutinans* and in naturally contaminated maize. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

The mycotoxin moniliformin has been structurally characterized as the sodium or potassium salt of 1-hydroxycyclobut-1-ene-3,4-dione. It was first isolated by Cole et al. [1] while screening for toxic products of a North American isolate of the fungus *Fusarium moniliforme* Sheldon cultured on maize. Subsequently, different *Fusarium* species were investigated for their moniliformin producing ability [2,3] and *F. subglutinans* (Wollenw. & Reink.) Nelson, Toussoun & Marasas, a common pathogen of maize and other cereal crops, was found to be the

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predominant producer. In a study of the incidence, geographic distribution and toxigenicity of *Fusarium* species in South African maize, *F. subglutinans* was found to predominate in relatively cool and humid climates [4]. The natural occurrence of moniliformin, in ears of hand-selected Transkeian maize visibly infected with *Fusarium*, was first reported by Thiel et al. [5] at concentrations as high as 25.0 mg/kg. *F. subglutinans* was also identified as the prevailing pathogen of maize ears in Poland, from 1985 to 1991, and the average moniliformin content over these six years was 130.9 mg/kg [6]. The levels of moniliformin in wheat, on the other hand, were found to be about 16 mg/kg on average. Moniliformin was also found to occur in Austrian maize with levels up to 20 mg/kg [7]. Results published by Sharman et al. [8] demonstrated the frequent presence of moniliformin as a cereal grain contaminant worldwide. Fungal contamination of corn and wheat poses a threat to human health, especially where these commodities form staple diets, as this toxin has been shown to cause acute focal myocardial degeneration and necrosis in experimental animals [9]. The mechanism of action probably involves selective inhibition of pyruvate and α-ketoglutarate dehydrogenase enzyme systems [10].

The synthesis [11] and spectroanalytical parameters [12,13] of moniliformin have been reported. However, its ionic nature presents considerable problems both in its selective extraction from different matrices and its adequate chromatographic separation on reversed-phase columns. As moniliformin is a highly water soluble and polar compound, it cannot be retained effectively with conventional mobile phases on a reversed-phase column. Thiel [14] used both ion-pair reversed-phase and ion-exchange liquid chromatography for the determination of moniliformin but reported that the moniliformin recoveries in the prepurification step were low and varied considerably. The first method required the use of tetrabutylammonium hydrogensulphate as the ion-pairing reagent in a methanol/sodium phosphate mobile phase while the second method made use of a strong anion-exchange column with 0.01 *M* sodium dihydrogen phosphate as the mobile phase. Another method employing ion-pair extraction and HPLC was reported by Shepherd and Gilbert [15] but the limit of detection was 0.1 mg/kg by UV detection and the procedure was reported by Sharman et al. [8] as being too time consuming for use in routine sample surveys. Improved methods by Scott and Lawrence [16] and Sharman et al. [8] were subsequently reported using UV detection. Scott and Lawrence used 40% (v/v) tetrabutylammonium hydroxide as the ion-pairing reagent and detection at 229 nm was found to produce a greater response than at 254 nm. A defatting step with hexane was necessary after extraction and levels down to 0.1 mg/kg were detectable in both wheat and maize. Sharman et al. [8] also used tetrabutylammonium hydroxide as the ion-pairing reagent but modified the clean up procedure for moniliformin cereal samples. The samples were cleaned up on a combination of reversed-phase and strong anion-exchange disposable cartridge columns. A limit of detection of 0.05 mg/kg was attainable, however, HPLC–UV analysis of naturally contaminated samples containing low concentrations of moniliformin is often hampered by background interference making the interpretation of chromatograms difficult. A more recent method using fluorescence detection following derivatization of moniliformin with 1,2-diamino-4,5 dichlorobenzene (DDB) was reported by Filek and Lindner [17], where the detection limit was reduced from 0.05 (UV detection) to 0.02 mg/kg maize. Despite these attempts to improve chromatography and detection limits, a more selective and sensitive detector is required to improve the analytical determination of moniliformin, since the acute and long-term toxicity of moniliformin in humans has not yet been fully investigated and the level of human exposure needs to be established.

Recent advances in LC–MS technology have shown this technique to have wide applications in food analysis [18–20]. MS detection eliminates the need for sample derivatization and moreover, has the potential to yield both structural and molecular weight information on analytes of interest. Furthermore, owing to its high power of mass separation, good selectivities can be obtained and, when used in the single ion monitoring (SIM) mode, increased sensitivities can be achieved as a result of decreased background noise. Using an established extraction procedure that has been shown to provide moniliformin recoveries in excess of 80% [16], this paper describes the use of HPLC–atmospheric pressure

chemical ionization (APCI) MS employing triethylamine (TEA) as a possible ion-pair reagent for the determination of moniliformin in strains of *F. subglutinans* and in naturally contaminated maize samples.

2. Experimental

2.1. Chemicals and solvents

Acetonitrile (HPLC grade), ammonium acetate (98% min) and ammonia solution (25% min) were obtained from Merck (Darmstadt, Germany) while TEA (99% min) was purchased from Sigma (St. Louis, MO, USA). Water for HPLC mobile phase was purified using a Milli-Q system (Millipore, Bedford, MA, USA). Moniliformin (as the sodium salt) was isolated and purified at PROMEC according to the method described by Steyn et al. [21]. Standard solutions were prepared in acetonitrile–water (95:5) and stored at 4°C.

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 the Centane district in the Transkei region during 1997. Two samples were collected randomly from household storage cribs, while another two were hand-selected as showing visible *Fusarium* infection.

2.3. Sample extraction and clean up

The method of Scott and Lawrence [16] was used for the extraction and clean up of moniliformin. Extracts from the cultures were prepared by homogenizing 20 g of culture material from each strain in 100 ml acetonitrile–water (95:5) for 5 min using a Polytron homogenizer (Kinematica, Luzern, Switzerland). The extracts were centrifuged on a Sorvall RC-3B refrigerated centrifuge (DuPont, CT, USA) at 4°C at 4000 *g* for 5 min and filtered into a 250 ml separatory funnel. The extract was partitioned with 150 ml hexane and the aqueous phase collected. A 5 ml aliquot of the partitioned extract was evaporated to dryness under nitrogen and the residue reconstituted into 250 μl methanol.

Extracts of naturally contaminated maize were similarly prepared. In brief, 25 g of sample was homogenized in 125 ml of extraction solvent and 80 ml of the aqueous layer was collected following partitioning with 150 ml of hexane. The aqueous layer was reduced under vacuum to a volume of approximately 4 ml before being transferred to a vial. Thereafter the extract was evaporated to dryness at 45°C under nitrogen and the residue reconstituted into 250 μl methanol. Moniliformin was previously reported to be fairly stable at temperatures below 50°C [16].

Clean up was performed using Bond Elut LRC C_{18} solid-phase extraction (SPE) cartridges (Varian, Harbor City, CA, USA) that were conditioned with 2 ml water. 250 μl of each extract was added onto the cartridges and moniliformin immediately eluted with 2 ml water. The eluate from each cartridge was evaporated to dryness at 55°C under a constant flow of nitrogen. The residues were redissolved in the HPLC mobile phase (500 μl) and diluted appropriately prior to injection into the HPLC. With this method of extraction and clean up, recoveries averaging 80% for maize and 85% for wheat were reported at spiking levels of 0.05 to 1.0 mg/kg, respectively [16]. Analytical results reported in this study were not corrected for recovery and are thus 15–20% lower than the true values.

2.4. Chromatography

HPLC analysis was carried out using a SpectraSeries P2000 pump equipped with a AS 1000 autosampler and a UV 1000 variable-wavelength UV detector (all from Thermo Separation Products, Riviera Beach, FL, USA). Moniliformin was separated isocratically on a 150×2 mm I.D. Luna C_{18} reversed-phase column (Phenomenex, Torrance, CA, USA) packed with 5 μm ODS-2. The mobile phase was prepared using 0.1 *M* ammonium acetate–

methanol–triethylamine (90:10:0.1) at pH 8.24 and pumped at 0.5 ml/min. The samples were filtered through a 0.45 μm syringe filter (Millipore, Yonezawa, Japan) prior to 20 μl injections onto the column. On-line UV detection at 229 nm was performed prior to MS detection.

2.5. Mass spectrometry

Negative ion APCI–MS was performed using a Finnigan MAT LCQ ion trap mass spectrometer (San Jose, CA, USA). The MS parameters were optimized by direct infusion of 30 μg/ml moniliformin standard at 5 μl/min into the source. The APCI vaporizer and mass spectrometer capillary temperatures were 350°C and 150°C, while the source current and source voltage were maintained at 5 μA and 8 V respectively.The capillary voltage was at −30 V, while the sheath and auxiliary gas flows were maintained at 70 and 30 units, respectively. During tuning, the mass spectrum was scanned from *m/z* 50 to *m/z* 150 whereas all subsequent experiments were performed in the SIM mode by monitoring the deprotonated molecular ion $[M-H]^-$ at *m/z* 97.

3. Results and discussion

3.1. MS tuning

Although for most molecules, positive-ion mode in APCI produces a stronger ion current, especially those with one or more basic nitrogen atoms, moniliformin is a highly acidic molecule and hence produces more negative than positive ions. Furthermore it is known that negative ion polarity mode sometimes generates less chemical noise than does the positive mode, thereby improving sensitivity. In addition moniliformin, being a low molecular mass compound, is difficult to detect amongst intense background signals, hence making negative ion detection appropriate. The negative ion APCI–MS spectrum obtained by continuous infusion of moniliformin standard directly into the source is shown in Fig. 1. Data was acquired in the ''profile'' mode and the full scan mass spectrum over the range *m/z* 50 to *m/z* 150 displayed the deprotonated molecular ion $[M–H]^-$ of moniliformin at *m/z* 97.

3.2. Mobile phase optimization

The mobile phase found most suitable for moniliformin analysis was a mixture of 0.1 *M* CH_3COONH_4–CH_3OH–TEA (90:10:0.1, pH 8.24). Ammonium acetate is a volatile buffer ideal for electrospray and has been extensively used in thermospray MS applications [22]. TEA provided moderate alkaline conditions compatible with column requirements. In addition, TEA also has ion-pairing capabilities. This two-fold advantage made TEA an effective mobile phase additive which together with ammonium acetate afforded greater retention of moniliformin (t_R=4.75 min).

3.3. Detection limit

In order to determine the on-column detection limit of moniliformin and the linearity of response of the deprotonated molecular ion versus the injected amount of analyte, different concentrations were injected and the single ion monitored. The precision of the measurement was readily determined by performing triplicate injections under identical conditions and found to have a RSD of 2.04% at the 16 μg/ml level. The minimum detectable amount injected was 10 ng (S/N=4). A linear fit with a correlation coefficient (R^2) of 0.9976 was observed for the MS signal from 1 μg/ml up to 34 μg/ml.

3.4. Analysis of strains of F. subglutinans

The five South African strains of *F. subglutinans* were analyzed for the production of moniliformin using this LC–MS method. Fig. 2A,B illustrate the single ion chromatogram obtained for each of the five strains. Extracts were diluted so as to yield responses within the experimental range of the calibration plot. Moniliformin levels ranged between 130 mg/kg and 1460 mg/kg culture. The strain MRC 115 was first isolated from maize in a high incidence area of esophageal cancer in Transkei and has been reported to produce high levels of moniliformin in cultures by several researchers [3,9,10]. Once again this strain was observed to be the highest producer of moniliformin, while MRC 1097 was shown to be a non-producer (<1 mg/kg). Nevertheless, in comparison to results reported by

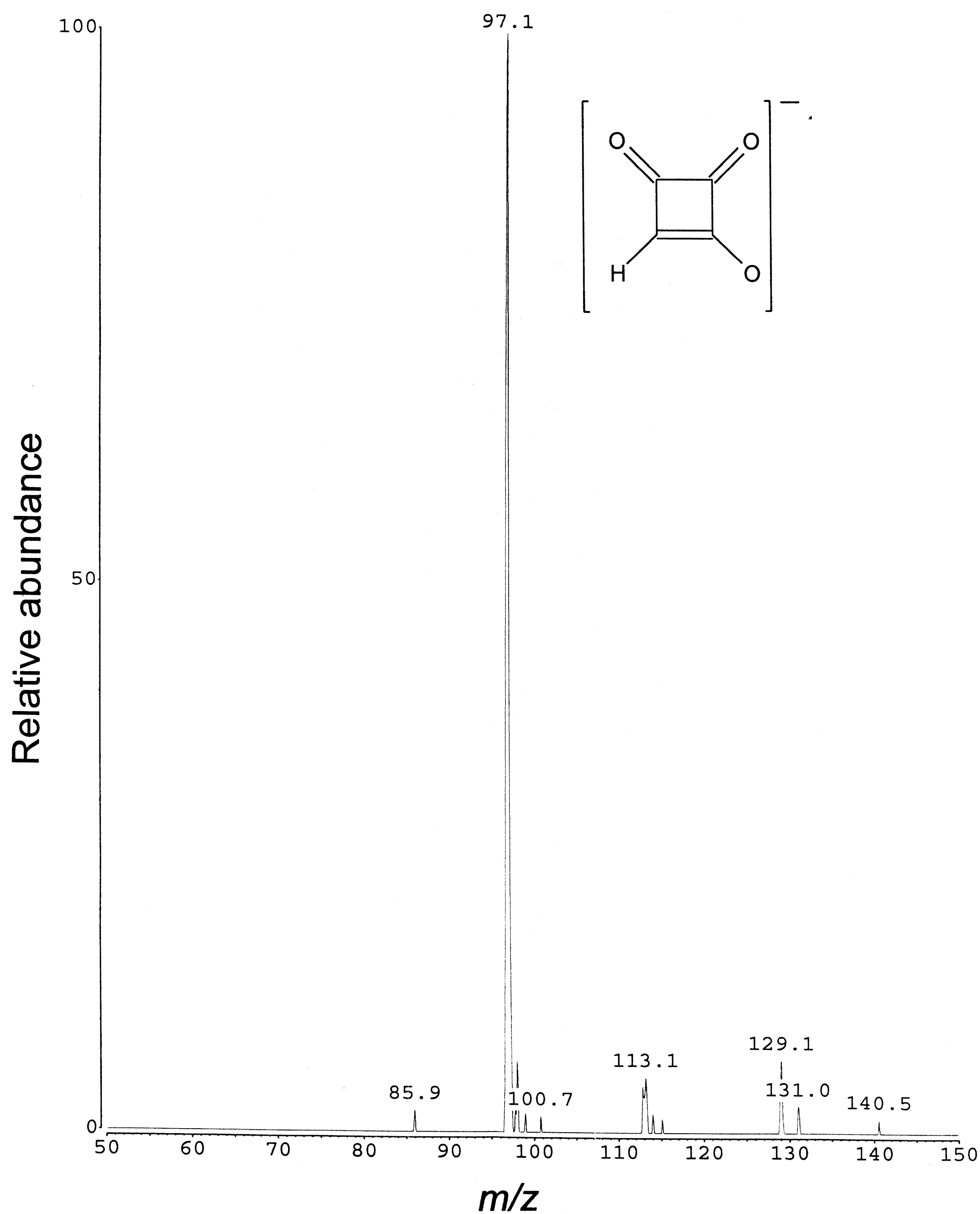

Fig. 1. Mass spectrum of moniliformin showing the deprotonated molecular ion $[M-H]^-$ at m/z 97.

Kriek et al. [9], MRC 115 which produced moniliformin in quantities up to 11.3 g/kg now produced approximately 10 times less. The use of TEA assisted in separating moniliformin from the other interfering ions of the same mass, hence making it applicable in such analyses.

In order to evaluate the agreement between UV and MS data, on-line UV detection was performed and moniliformin concentration calculated using both detection techniques.

The quantitative data were plotted against each other and compared well (R^2=0.9997). One nevertheless needs to be aware that when analyzing maize extracts, matrix effects can pose problems in quanti-

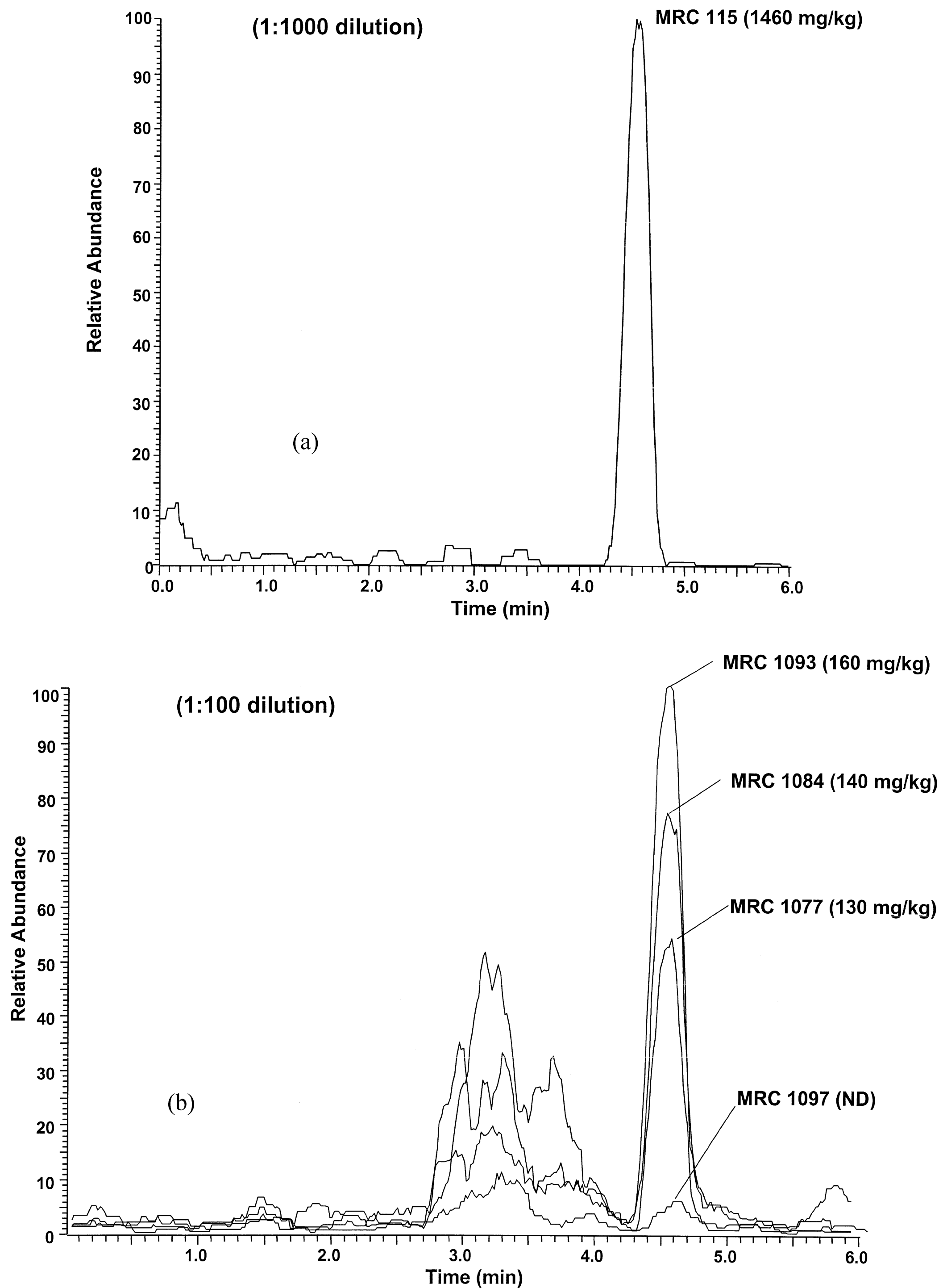

Fig. 2. Single ion chromatogram of moniliformin in *F. subglutinans* culture material. For experimental conditions, see Sections 2.4 and 2.5. (a) MRC 115 (1:1000 dilution), (b) MRC 1093, MRC 1084, MRC 1077 and MRC 1097 (1:100 dilution)

tation. In this case, mass spectral data can prove more credible that UV data.

3.5. Analysis of naturally contaminated maize

The technique was further applied to naturally contaminated maize samples obtained from Transkei, South Africa. Four samples were collected of which two were randomly sampled from storage cribs (D1A, D2A) while the other two were selected as showing visible *Fusarium* infection (D7B, D9B). Moniliformin was not detected (i.e. <5 μg/kg) in randomly selected samples but were present at levels 17 μg/kg and 10 μg/kg (RSD=8.2%) in samples D7B and D9B respectively. While only four samples were analyzed, this nevertheless reveals that there may be widespread exposure to low levels of moniliformin through moldy maize consumption. The use of LC–MS makes the determination of such low levels possible, which is currently not easily attainable with other instrumental methods.

4. Conclusion

The LC–MS method was optimized to yield enhanced sensitivity and specificity for moniliformin determination in both dried culture material and naturally contaminated maize. The use of ion-pairing reagent assisted in improving the retention capabilities, making separation of moniliformin from interfering compounds possible. This extraction technique, coupled by this sensitive analytical method, makes possible the assessment of potential risks associated with the low but chronic consumption of moniliformin contaminated maize. With this method we were able to detect quantities as low as 10 μg/kg of maize.

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