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METHOD FOR THE ANALYSIS IN MAIZE OF THE *FUSARIUM* MYCOTOXIN MONILIFORMIN EMPLOYING ION-PAIRING EXTRACTION AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The *Fusarium* mycotoxin moniliformin (hydroxycyclobutenedione) has been determined in maize using a novel method with a recovery of 70–80% at 400–1600 μg/kg and 60% at the detection limit of 100 μg/kg. The method requires extraction of the toxin into aqueous tetra-*n*-butylammonium hydroxide and removal of cations from this solution by ion-exchange chromatography. Following clean-up by partitioning against dichloromethane, further quaternary ammonium reagent was added to the aqueous phase which was absorbed onto a hydrophilic matrix and the tetra-*n*-butylammonium moniliformate ion pair extracted into dichloromethane. After evaporation of the organic eluent, the residue was dissolved in aqueous sodium chloride and moniliformin quantitated by ion-pair high-performance liquid chromatography with UV detection. A batch of five samples may be analysed in 5–6 h including the chromatographic determination.

INTRODUCTION

Moniliformin, a mycotoxin containing the cyclobutene skeleton, was first isolated as a *Fusarium* metabolite by Cole *et al.*[1], who later characterised the toxin by X-ray crystallography[2] and described its chemical synthesis[2]. Other synthetic routes are available[3,4] and the compound may also be obtained readily by biosynthesis[5,6]. Only a limited number of *Fusarium* species are capable of producing moniliformin[7,8] but yields of up to 33.7 g/kg of substrate have been recorded[7] and the species involved are reported as common pathogens of maize in most maize-producing areas of the world[9].

The high incidence of human oesophagal cancer in the Transkei has been correlated with the presence in staple maize of a number of potentially toxigenic *Fusarium* species[10]. In addition, certain diseases of animals are known to be associated with the consumption of mouldy maize. Although not solely responsible for these diseases, moniliformin is a highly toxic *Fusarium* metabolite[1,11–13] and it is important therefore that sensitive and reliable methods of analysis be available for this com-

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pound. Current methods are however not entirely satisfactory. Kriek *et al.*[11] were able to assay moniliformin at high concentration in cultures by a simple solvent extraction with subsequent UV analysis of the solution. This method is slow, insensitive and subject to interference, despite the use of wavelength ratioing for confirmation. These and other workers have used thin-layer chromatography (TLC) for analysis, visualising the toxin by quenching of fluorescence[6] or alternatively by reaction with 2,4-dinitrophenylhydrazine (2,4-DNP)[12,15], ninhydrin[1,5] or N-methylbenzthiazolon-2-hydrazone (MBTH)[16].

TLC detection limits for moniliformin standards are typically in the range 0.1–1 μg, but for sample extracts limits are very dependant upon clean-up and are usually greater than 1 mg/kg. Kamimura *et al.*[15] were able to reduce this to 100 μg/kg but at the expense of a lengthy purification and derivatisation procedure followed by final quantitation by TLC and subsequent densitometry. The recovery reported was 76% at 2 mg/kg added toxin. Thiel *et al.* employed ion-pair high-performance liquid chromatography (HPLC)[14] with UV detection at 227 nm but the sample clean-up was time consuming and gave low and variable recoveries. Direct HPLC analysis of the initial aqueous extract was preferred[14] but with a detection limit which may be estimated at *ca.* 10 mg/kg from the published chromatograms[14]. The TLC method of Jansen and Dose[16] with MBTH as a visualising reagent had a sensitivity of 75 ng of moniliformin on the plate but the detection limit in food samples was not reported. Recoveries of 51–97% were indicated for moniliformin added to rice at concentrations of 2–20 mg/kg.

This paper describes a sensitive and relatively rapid method for moniliformin in maize which takes advantage of the acidic nature of the toxin to employ ion-pairing phenomena not only for the final analysis but also for the preliminary sample clean-up. A similar approach is in principle applicable to the analysis of other ionic compounds, including mycotoxins such as tenuazonic acid, cyclopiazonic acid and the ochratoxins.

EXPERIMENTAL

Materials

Moniliformin was purchased as the sodium salt from Sigma (St. Louis, MO, U.S.A.) and the free acid was custom-synthesised by Lancaster Synthesis (Morecambe, U.K.). All toxin samples were held at −36°C. Commercial maize samples were obtained from a number of sources. Tetra-*n*-butylammonium hydroxide (TBAH) was purchased as a 40% (1.5 *M*) aqueous solution (Lancaster Synthesis). Tetra-*n*-butylammonium dihydrogen phosphate (TBAHP), 1.0 *M* solution in water, was from Aldrich (Poole, U.K.) and Celite 545 filter aid from Koch-Light (Haverhill, U.K.). Amberlite IRC 50 (H^+), analytical grade (14–52 mesh BSS) from BDH (Poole, U.K.) was equilibrated before use with 1 *M* hydrochloric acid and washed with water until the aqueous supernatant was neutral. C_{18} "Sep-Pak" cartridges were purchased from Waters Chromatography Division of Millipore (Bedford, MA, U.S.A.).

Fritted glass (porosity 1) chromatography columns (300 × 22 mm I.D.) were from Soham Scientific (Ely, U.K.). ChemTubes (CT 2050, 50-ml capacity) were obtained from Analytichem International (Harbor City, CA, U.S.A.). HPLC columns were purchased from Hichrom (Reading, U.K.). Filter membranes (LC13, 0.2 μm),

for clarifying samples immediately prior to HPLC analysis, were from Gelman (Ann Arbor, MI, U.S.A.). Water for HPLC mobile phases was purified in a Milli-Q system (Millipore, Bedford, MA, U.S.A.). All other solvents were HPLC grade from Rathburn Chemicals (Walkerburn, U.K.).

Methods

Purification of TBAH. The quality of this reagent varied significantly between batches and it was purified if necessary by partition against two equal volumes of dichloromethane, which removed the greater part of the yellow colouration of the material supplied. Some lots were completely miscible with dichloromethane and addition of water (up to 20% by volume of the TBAH reagent) was required to promote phase separation. Dichloromethane was removed from the purified reagent by bubbling a stream of nitrogen through the solution.

Extraction. Ground maize (30 g), water (149 ml) and TBAH (1.0 ml, *ca.* 1.5 mmol) were placed in a 250 ml Ehrlenmeyer flask and swirled on an orbital shaker at 200 rpm for 30 min. This slurry was passed under suction through a 5-mm layer of Celite in a Buchner funnel and the filtrate reserved.

Clean-up. A bed (80 × 22 mm I.D.) of Amberlite IRC 50 was slurry packed in water in a glass column and the whole filtrate was poured onto the column (taking care to minimise dilution of the filtrate with water contained within the resin bed) and passed through at a rate of *ca.* 3 ml/min, the first 40–50 ml of eluate being discarded. The next 50 ml were collected, and passed rapidly through a C_{18} "Sep-Pak" previously primed with acetonitrile. TBAH (0.1 ml) was added, and the mixture swirled and poured into a 50 ml capacity ChemTube. This was eluted with 4 × 50 ml dichloromethane (two additional volumes above those specified by the ChemTube manufacturer) and the combined organic extracts evaporated to dryness under vacuum. The residue was transferred with dichloromethane to a 2 ml vial (having a screw cap and a PTFE-faced septum) and the solvent removed under a stream of nitrogen. The residue was dissolved in 0.10 ml acetonitrile and 0.30 ml 50 m*M* aqueous sodium chloride added for isocratic ion-pair HPLC determination of moniformin, giving a final residue concentration of 0.5 gram equivalents of maize in 20 μl of solution. Before analysis, the solution was passed through a 0.2-μm membrane to remove particulates.

HPLC. The system employed consisted of a Varian Assoc. 5500 liquid chromatograph, a Rheodyne 7125 injection valve with a 20-μl loop and a Pye LC-UV detector fitted with an 8-μl flowcell and set at 260 nm. The column (Spherisorb 5 μm ODS, 250 × 4.9 mm I.D.) was thermostatted at 35°C and equilibrated with mobile phase (acetonitrile–10 m*M* aqueous TBAHP, 25:75) for at least 15 min before analysis. At a flow-rate of 1.0 ml/min, toxin retention time was 7.7 ± 0.2 min, depending upon the degree of equilibration. Samples were injected about every 20 min. The column was flushed out with 100% acetonitrile as necessary.

RESULTS AND DISCUSSION

HPLC was chosen as the analytical method for this determination primarily because of the improved resolution and ease of quantitation when compared with TLC. The HPLC determination of mycotoxins has recently been reviewed[17]. In the

reversed-phase mode of HPLC moniliformin was eluted at the column dead volume even with 100% water as the mobile phase. However, ion-pair HPLC has previously[14] been shown to allow retention and thus separation of the toxin. These results may readily be explained in terms of the acidic character of moniliformin, which has been reported[5] as having a pK_a of 1.7. Under the ion-pairing HPLC conditions employed here, the minimum detectable mass of moniliformin standard was 10 ng.

As usual, the sensitivity attainable in a foodstuff depends primarily upon the efficiency of sample clean-up because this is the major limiting factor upon the mass equivalent of food injected into the column. The Kamimura *et al.*[15] clean-up entails several evaporations of solutions of toxin in water and it has been our experience that this operation can give rise to extensive losses of moniliformin. Recoveries from aqueous or mixed organic-aqueous solutions taken to dryness or near-dryness were low or nil. Thiel *et al.* reported low and erratic recoveries from a method incorporating lyophilisation[14]. Thus a desire to avoid the removal of water led us to consider how the ion-pair phenomenon successfully utilised for HPLC analysis could also be employed for the prior isolation and clean-up of moniliformin from maize.

It was established that moniliformin could not be extracted from water by dichloromethane but addition of tetra-*n*-butylammonium counter-ion led to complete transfer of toxin to the organic phase. Parition of the ion-paired toxin from an aqueous maize extract was however for some samples less efficient, probably owing to the competitive effect of non-organic-soluble cations from the cereal. Preliminary removal of cations by ion-exchange chromatography (IEC) circumvented this problem. A weak (carboxylate-based) exchanger was preferred because it had previously been found that exposure of moniliformin to low pH led to loss of the toxin. Fig. 1 shows chromatograms of residues from a maize spiked at 800 μg/kg, with the extract either taken through the complete clean-up procedure (Fig. 1a) or loaded directly onto a "Chem-Tube" without prior ion exchange or Sep-Pak treatment (Fig. 1b).

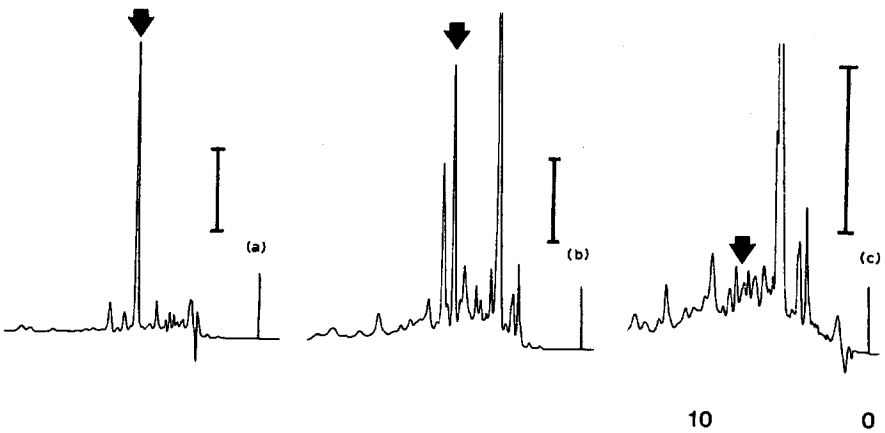

Fig. 1. Comparison of full and abbreviated clean-up procedures: (a) and (b) extract of blank maize spiked with 800 μg/kg moniliformin and cleaned up by either the full procedure (a) or abbreviated procedure (b); (c) blank maize —abbreviated clean-up. Detector set at 260 nm. Bar represents 0.04 O.D. units. Analytical conditions as in Methods.

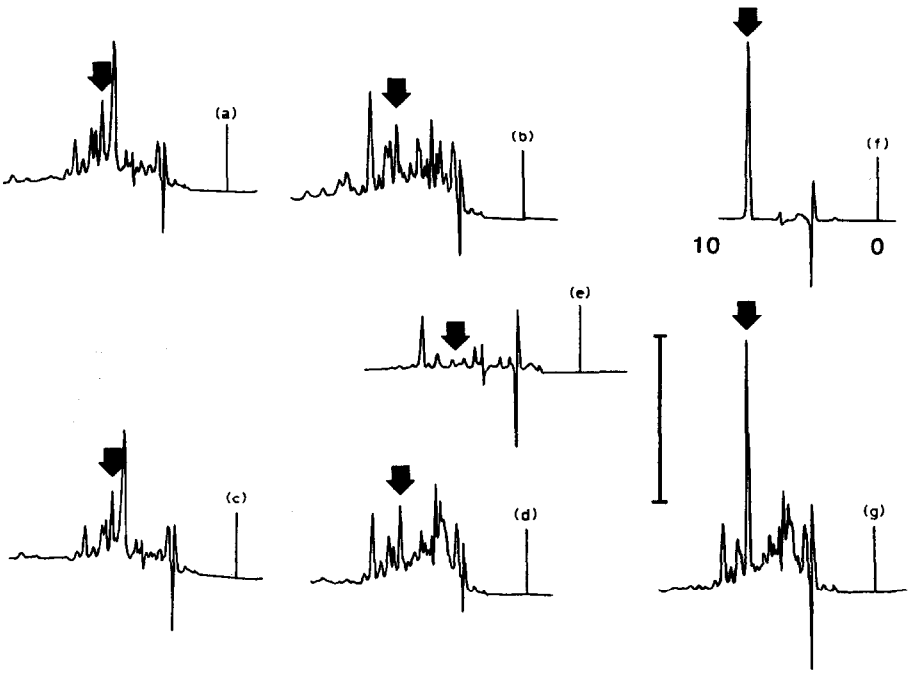

Fig. 2. Chromatograms of blank maize extract purified as indicated after preliminary extraction with aqueous TBAH and ion exchange. (a) After clean-up by dichloromethane partition only; (b) no additional clean-up; (c) clean-up by both dichloromethane partition and also C_{18} Sep-Pak; (d) clean-up only by C_{18} Sep-Pak. (e) Reagent blank (water sample) after both additional treatments; (f) 100 ng of moniliformin standard; (g) final residue from (d) with 100 ng (200 μg/kg equivalent) of moniliformin added. Bar represents 0.04 O.D. units. Analytical conditions as in Methods.

This "abbreviated" cleanup, although less specific, is reasonably effective for heavily contaminated samples. The recovery achieved with the abbreviated cleanup is *ca.* 10% less than that found after the full treatment, probably for the reasons outlined above. Fig. 1c shows the blank maize analysed after the abbreviated clean-up. There is only a trace level peak at the moniliformin retention time, equivalent to perhaps 20 μg/kg, although after the full clean-up a concentration of 100 μg/kg was consistently found (see Fig. 2 and discussion of confirmation by wavelength ratioing).

In preliminary experiments the maize extract after IEC was partitioned against dichloromethane (before addition of tetra-*n*-butyl-ammonium ion), in order to remove potential chromatographic interferences. An emulsion formed which could be broken either by centrifugation or by separation overnight. No loss of recovery was encountered in the later case. However, the improvement in clean-up was minimal (see Fig. 2) and this time-consuming step was eliminated in the final method. Additional purification with a C_{18} "Sep-Pak" was required only where samples contained moniliformin at concentrations below 200 μg/kg. Formation of an emulsion during the dichloromethane partition after addition of TBAH was avoided by employing a "Chem-Tube" column at this stage, but it was found that extraction with four aliquots of dichloromethane (and not two as recommended by the manufacturer) was required to obtain a satisfactory recovery of ion-paired moniliformin from maize extracts. When 8 μg of toxin were added to aqueous TBAH and loaded onto a

"Chem-Tube", only 40% was found in the first two aliquots, with 35% in the third and 10% in the fourth aliquot.

Recovery from maize was determined by adding toxin in solution to the cereal and removing the solvent at ambient temperature for 30 min under a purge of oxygen-free nitrogen. Where the toxin was added as the sodium salt, recoveries of 80% at the 200 μg/kg level were attainable using pure water as the extractant. However, it had been found elsewhere that when maize was spiked with an acetonitrile solution of the free acid form of moniliformin, essentially none of the toxin was extracted into water[18], a result which was confirmed in this laboratory. Extraction with methanol–water (20:80) was evaluated but this was unsatisfactory because of extensive interferences in the final residue. The use of aqueous TBAH gave acceptable recoveries (Table I) without interferences (although much more material was seen to be extracted from the maize) whether the sample was spiked with the free acid or the sodium salt. The addition of more TBAH at either initial extraction or "Chem-Tube" stages did not improve the recovery.

The recovery of moniliformin from aqueous TBAH itself (pH 12) was poor (17% of 12 μg added to 149 ml of water and 1 ml of TBAH and treated as a cereal sample) and this was attributed to instability of the toxin under basic conditions. The presence of maize coextractives buffered the mixture to pH 6, and moniliformin was stable for at least 18 h in cereal extract containing TBAH. Ion-exchange treatment of the extract reduced the pH to 4.5 but the stability of the toxin in this case was not evaluated. Only a few maize samples have been analysed so far and the choice of extraction solvent will be re-examined when a suitable naturally-contaminated maize sample has been found. Final residues in solution were stable at ambient temperature for at least three days.

UV detection at 260 nm was employed because although the ratio of extinction coefficients of moniliformin at 228 and 260 nm is reported[19] as 3.4, absorption at the lower wavelength is less selective and interferences are more likely. However, no evidence was found for interferences in the limited number of maize samples analysed. Fig. 3a and b show chromatograms of the same residue from a blank maize, with the detector set at either 260 nm (Fig. 3a) or 228 nm (Fig. 3b). Similarly, Fig. 3c and d shows chromatograms at 260 and 228 nm of the residue from this maize spiked at 200 μg/kg with the free-acid form of moniliformin before extraction. The absorption ratio (not compensated for solvent absorption) found in both cases was 3.6–3.7, while that of a 0.4 μg standard was 3.5. Thus wavelength ratioing may be

TABLE I

RECOVERY OF MONILIFORMIN ADDED TO MAIZE

Concentration of toxin added (μg/kg)	*Added as sodium salt recovery (%)*	*Added as free acid recovery (%)*
100	61, 59	51
200	65, 69, 84	70
400	82	67, 82
800		64, 74
1600		74

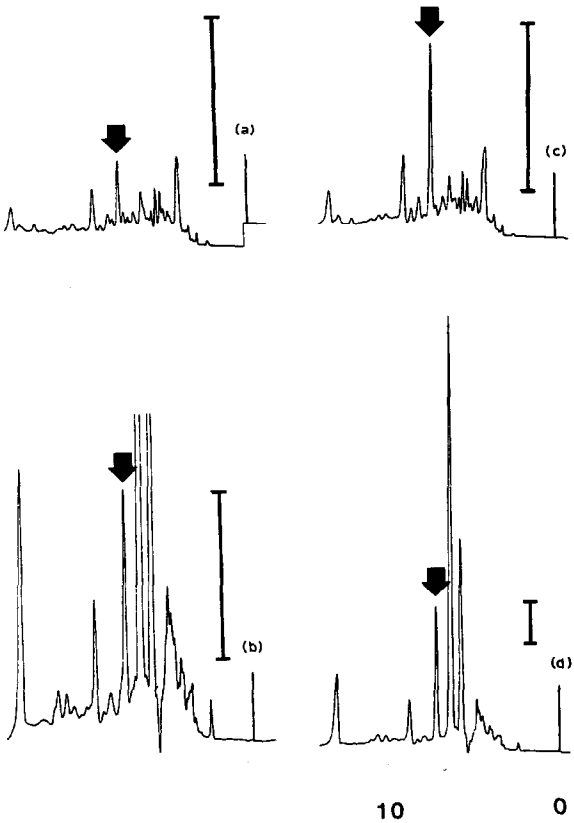

Fig. 3. Chromatograms of (a) and (b) blank maize residue after full clean-up chromatographed with detector set at (a) 260 nm or (b) 228 nm. (c) and (d) Chromatograms of blank maize spiked at 200 μg/kg with moniliformin free acid and carried out through complete clean-up. Detector set at (c) 260 nm or (d) 228 nm. Bar represents 0.04 O.D. units. Analytical conditions as in Methods.

employed for confirmation, along with co-chromatography of residues with standard toxin. Other confirmation techniques require development.

The method as described permits the determination of moniliformin in maize at 100 μg/kg with acceptable recovery of added toxin. The total time required for clean-up and analysis of a batch of five samples is *ca.* 5–6 h. It may be acceptable to employ the abbreviated (and more rapid) cleanup when screening for samples containing in excess of *ca.* 500 μg/kg toxin but further evaluation of recovery is required in this case. However, when using this very simple method extra care is required to ensure the purity of the TBAH reagent because more than three times as much TBAH is loaded onto the Chem-Tube when the ion-exchange step is omitted. Some batches of reagent were deep yellow and much of this coloured material was carried through the clean-up, causing extensive interferences in the final residue. Purification of the reagent by preliminary extraction with dichloromethane resulted in substantial improvements but the final material remained faintly coloured. The base was not re-standardised after extraction although a considerable reduction in TBAH concentra-

tion in the aqueous reagent was obvious from the mass of material found on evaporation of the dichloromethane layer. Another sample of TBAH was prepared by anion-exchange chromatography of TBADP and this was almost colourless. The reagent blank in Fig. 2 was obtained with doubly-extracted TBAH. The detection limit of 100 μg/kg is set by potential interferences from coextractives and could possibly be reduced further at the expense of additional clean-up of the final residue, perhaps using other types of reversed-phase cartridges.

Current work in this laboratory includes a small survey of maize imported into the U.K., evaluation of TLC as a screening technique and the development of gas chromatography–mass spectrometry and liquid chromatography–mass spectrometry confirmation methods. It is also intended to apply the principle used in this analysis to the determination of other ionic compounds, including mycotoxins such as ochratoxin A, tenuazonic acid and cyclopiazonic acid.

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