MYCOTOXIN PRESENCE IN FOOD PRODUCTS

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Abstract

Fusarium moniliforme produces metabolite fusarin C with mutagenic risk for humans and mamals. A new method has been developed for determination of fusarin C in cereal products by liquid chromatography (LC) with UV detection; small silica gel or bonded phase amino (NH2) columns of 3 ml capacity are used for cleanup and laboratory light is restricted. We study stability of fusarin C in stress conditions. The mutagenicity of those metabolites was lower in thermal treated corn products. There is no fusarin C in corn studied samples that contained deoxynivalenol, but it was detected in unsterilized field corn incubated in the laboratory. Thin layer chromatography (TLC) and solvent stability of moniliformin, another important mycotoxin from F. moniliforme, have been recorded at high levels.

Key words: Fusarium moniliforme, fusarin C, mycotoxins, moniliformin

INTRODUCTION

F. moniliforme was one of several fungal species with a higher level of contamination of cereals in counties in Henan province, China that had a hugh incidence of esophageal cancer compared to counties with a low incidence. Experimental studies in South Africa and in China have shown that cultures of *F. moniliforme* on corn and corn products can cause cancer in rats; the hepatocarcinogen(s) produced by one strain can survive drying of the corn culture at 45-50º for 24 h. Furthermore, at least two classes of mutagens may be formed by *F. moniliforme. Fusarium moniliforme* is a fungal parasite of a wide variety of plants and, in particular, is one of the most important pathogens of corn (maize) in the world. The incidence of *F. moniliforme (F. verticillioides)* in corn has been correlated with human esophageal cancer rates in Transkei.

Spontaneous conversion of fusarin C and its stability in ultraviolet (UV) light and in aqueous solution at different temperatures have also been studied. Although carcinogenicity testing of fusarin C has not yet been reported, its mutagenic activity is comparable to that of aflatoxin B_1 and sterigmatocystin. In view of all the foregoing observations, development of a rapid method for determination of fusarin C in foodstuffs and further stability studies related to food preparation were clearly needed.

Fusarium moniliforme has been associated with several animal mycotoxicoses and can produce a variety of toxic metabolites in addition to

fusarin C. One of these is moniliformin, which occurs in corn cultures of *F. moniliforme* and *F. subglutinans* (*F. moniliforme var. subglutinans*) as the potassium and/or sodium salt; the pk_a of the free acid is low (0.0 – 1.7) yields as high as 3.4% have been found. Moniliformin is also formed by other *Fusarium* species.

Densiometric thin layer chromatography (TLC) was used in two methods for analysis of moniliformin in grains and these are the only two methods for which recovery data are given; extraction solvents were methanol and methanol-water (95+5).

MATERIAL AND METHODS

Methods applied to analysis of fusarin C in corn and wheat products consisted of extraction, cleanup by column chromatography, and high performance LC. Variations used in each of these procedures are designated below. Analyses were carried out under "gold" fluorescent lighting.

Extraction

The sample (10 g ground corn, corn meal, of wheat flour) was blended for 5 minutes with 1) 100 ml methylene chloride-acetonitrile (1+1) or 2) 100 ml acetonitrile. The extract was filtered, and 50 ml was evaporated under reduced pressure at 30ºC. The extraction procedure was conveniently carried out on 1/10 scale for heat stability studies.

Cleanup

A) The sample residue was dissolved in 2 ml methylene chloride-methanol (97+3) and 1 ml added to a column (1.5 cm i.d.) of 5 g silica gel 60 slurried in the same solvent mixture. The column was washed with 60 ml methylene chloride-methanol (97+3) and fusarin C eluted with 50 ml (30 ml for muffins) of methylene chloride-methanol (9+1). This eluate was evaporated under reduced pressure (30 ºC) and dissolved in LC mobile phase.

B) Degassed solvents were used for chromatography on small pre-packed columns. The sample residue was dissolved in 1-2 ml methylene chloride-methanol (99+1) and 0.1 to 0.5 ml (max 1.25 g sample equivalent) addes to a 3-ml amino (NH_2) bonded phase disposable pre-packed column previously conditioned by washing with 1 ml of the same solvent. The column was washed with 2.25 ml of this solvent, then fusarin C was eluted with 1.5 ml methylene chloride-methanol (9+1). The eluate was evaporated in a vial under nitrogen without heating, dissolved in LC mobile phase anf filtered.

C) Procedure B) was also carried out using 3-ml silica gel disposable pre-packed column, except the residue was dissolved in methylene chloride-methanol (97+3), the column was washed with 3 ml of

this solvent, and fusarin C was eluted with 3 ml methylene chloride-methanol (9+1).

High performance LC

(i) Reverse phase LC was carried out on a 250 x 4.6 mm 5µm actadecylsilane column (Altex Ultrasphere or HPLC Technology Ltd. Ultra Techsphere); mobile phase was methanol-water (7+3) at a flow rate of 1 ml/min; UV absorption at 365 nm (Waters model 440) was used for detection.

(ii) Normal phase LC – a 250 x 4.6 mm ZORBAX SIL (5-6 µm) column (DuPont) was used with methylen chloride-methanol (95+5) mobile phase (1 ml/min) and 365 nm detection. The equivalent of 2-20 mg sample was injected for both (i) and (ii) (20 µL loop).

The preferred methods for analysis of fusarin C in cereal products comprised extraction solvent (2) (acetonitrile), cleanup procedure (B) or (C), and normal phase LC (2(B) and 2(C). The limit of detection was about 0.05 µg/g. Other variations of procedures are quoted as they were also used in studies reported here.

RESULTS AND DISCUTIONS

STABILITY of FUSARIN C

Standards

Three preparations of fusarin C were used which were stored as gums at -4ºC. The first one A1 was, received in september 2010 from SCDA Oradea; the second A2 was prepared in laboratory in December 2010 by column chromatography and preparative TLC from an ethyl acetate extract of *Fusarium moniliforme* grown on Myro liquid medium; the third A3 was similarly obtained inn late november 2010 from pooled methylene chloride-acetonitrile (1+1) extracts of several *F. moniliforme and F. graminearum* strains grown on corn. UV spectra of freshly prepared A1 and A2 had ϵ_{359} (in methanol) of 26,258 and 29,297, respectively. However, storage of fusarin C in the freezer resulted in extensive decomposition, which was not apparent qualitatively by LC analysis. The material from sample A1 had $_{E}350$ of only 6,299 in December 2010 and $_{E}348$ of 4,195 another 126 days later (with a second UV absorption maximum at 291 nm). The rate of decomposition of the fusarin C in this already impure material, as indicated by the longwave UV absorption, was slower than that of freshly isolated A1, an observation borne out by the change in the ratio of the LC peak heights over a similar time period.

TABLE 1

Recovery of fusarin C from cereal products

Method variation	Sample	Fusarin C Added (μg/g)[a]	Recovery (%) [b]
1 (A) (i)	Corn flour	0.11	92 (4)
"	"	0.12	84 (2)
"	"	0.17	84 (4)
"	"	0.32	89 (6)
"	"	1	94 (5)
"	"	3,21	79 (1)
2 (A) (i)	Corn meal	43,11	117 (1)
1 (B) (i)	Ground corn	0.2	100 (1)
1 (B) (i)	"	2	95 (2)
2 (B) (ii)	Corn meal	20	87 (1)
2 (B) (ii)	Wheat flour	20	82 (2)
1 (C) (i)	Ground corn	2	95 (1)
2 (C) (i)	Corn meal	1	97 (2)
2 (C) (ii)	Corn meal	20	92 (2)
"	"	1	85 (2)

[a] Spiking concentration uncorrected for standard decomposition.

[b] Number of samples analyzed given in parentheses.

The nature of the decomposition product(s) is not known; they do not appear to correspond to previously reported spontaneous decomposition products. A more polar TLC spot was detected in the 4 month old A1 material on spraying and heating the plate with acidic anisaldehyde, a critical purity test for fusarin C. In view of these problems, it is apparent that concentrations of fusarin C quoted are not always absolute, particularly for the recovery studies with method 1(A) (i) and solution studies (see below).

Exposure to fluorescent laboratory lighting of methanol-water (7+3) solutions of fusarin C in 4-ml glass vials placed on the laboratory bench caused rapid degradation. Fusarin C is known to be highly sensitive to UV light and the less polar product formed could P ' 1 or P ' 2. A plastic UV

filter sleeve placed round the fluorescent lamps was not completely satisfactory in preventing slow decomposition of fusarin C. However, negligible losses resulted when the lights were replaced with "gold" fluorescent lights. As a further precaution, vials were wrapped with aluminum foil, which was also placed over the flask and water bath of the rotary evaporator.

FUSARIN C presence

Twelve samples of ground corn from the SCDA Oradea were analysed for fusarin C by method 1 (A) (i), with negative results. These samples all contained deoxynivalenol. A corn cob obtained from privat farmer and incubated at 28ºC (70% relative humidity) for 1 week was found to contain 1.5 µg/g of fusarin C (value uncorrected for standard deterioration) whereas a control unicubated corn cob was negative.

MONILIFORMIN presence

Preliminary to development of a method for determination of moniliformin, we investigated its TLC behaviour using 3-methyl-2-benzothiazolinone hydrazone (MBTH) hydrochloride as spray reagent and also its stability (free acid and salt form) in some common solvents (Table 3).

TABLE 2

Effect of corn meal extract on mutagenicity of fusarin C (4 µg/plate)

Corn meal Mg/plate	Revertants/plate	
	Unheated Corn meal	Heated Corn meal [b]
200	1633	1514
600	1821	1625
1,000	1785	1592
2,000	732	222

[b] 100 ºC for 1 hour.

TABLE 3

Stability of moniliformin (1 mg/ml) in several solvents

Solvent	Free acid	Sodium salt
Methanol	Unstable: 2 high Rf spots formed within 1 day.	Soluble. Stable 2 months/22 ºC or 1 hour/65 ºC
Ethanol	Unstable: 2 different high Rf spots formed.	
Water	Solution slowly goes yellow	Soluble and stable
Acetone	Minor, less polar product, formed (2 days).	Not completely soluble; need to add water (30%). Stable.
Acetonitrile	Stable (2 months)	Not completely soluble.
Methylene chloride	Only partly soluble	

CONCLUSION

Fusarin C was moderately stable in ground corn when stored in the dark for up to 7 days at room temperature: recoveries averaged 57% 1-7 days after spiking (0.5 µg/g) compared to the control recovery of 80%. A temperature of 100ºC caused marked decomposition of fusarin C when added to corn meal (Fig. 6), although no significant new peak could be detected by LC. Similar results were obtained with wheat flour. An extract of heated corn meal showed almost complete loss of mutagenicit, which was not due to any possible antimutagenic effect ff the extract of heated corn meal. Complete loss of fusarin C was observed by LC on baking corn meal muffins for 20 minutes at 230 ºC after spiking the mix with 2 µg/g (dry weight).

The latter study of monoliformin presence was particularly relevant as the free acid is being distributed as a standard, and as shown in Table 3, it is unstable in alcohols. For method development the potassium salt was used. Several solvents were tried for its extraction from spiked corn meal, with generally low recoveries. However, acetonitrile-water (84+16) gave 60-80% recoveries (4 and 20 µg/g). For TLC spot comparison, an initial development of the chromatogram with t-butyl ether before development with chloroform-methanol-formic acid (70+30+0.16) (equilibrated) was advantageous; the detection limit was about 25 ng.

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