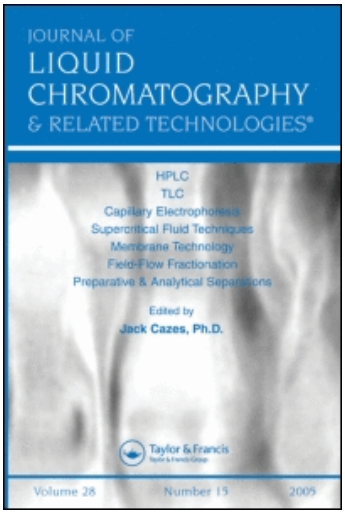

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DETERMINATION OF STERIGMATOCYSTIN IN FUNGAL CULTURES BY HIGH PRESSURE LIQUID CHROMATOGRAPHY

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ABSTRACT

Levels of sterigmatocystin in cultures of various fungal strains were determined by high pressure liquid chromatography (HPLC). This mycotoxin was well separated using acetonitrile / water / acetic acid (55/45/2) as solvent on a μ-Bondapak C18 column (Waters Associates). It was detected by its absorbance at 320 nm. Using this method, sterigmatocystin can thus be simply and rapidly determined in mould strain cultures.

INTRODUCTION

Since the discovery of aflatoxins, contamination of human food or animal feed by mycotoxins has been widely documented. Many workers have investigated the possible involvement of other toxic mould metabolites, such as sterigmatocystin, in human and veterinary toxicology (1,2,3,4).

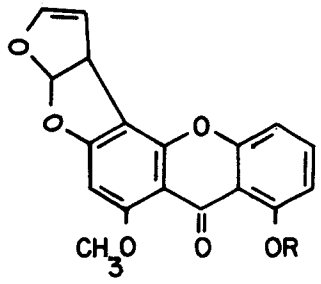

Figure 1

Sterigmatocystin : structure a : R = H ; b : R = $COCH_3$

The chemical structure of sterigmatocystin is shown in figure 1 (5), and it has been shown to be both carcinogenic and generally toxic (6,7,8,9). It is produced in high yield by various strains of commonly encountered moulds such as Aspergillus versicolor, Aspergillus nidulans, by an unidentified species of Bipolaris (10), as well as by Aspergillus flavus, Aspergillus chevalieri, Aspergillus ruber and Aspergillus amtelodami (11).

Several methods for the determination of sterigmatocystin using thin layer chromatography have been described (12,13), and methods are available based on gas-liquid chromatography (GLC) (14,15,16).

However, all these methods have disadvantages. They are either time consuming, have a low sensitivity or present problems in extract quantitation. In addition, for the techniques using GLC, there is the problem of cost, as the detection methods are usually based on mass spectrometry.

Lepom (17) has recently described a new method for determination of sterigmatocystin in animal feed by high performance liquid chromatography (HPLC) using column switching. This method has a higher sensitivity than methods based on gas-liquid chromatography, although it is somewhat difficult to setup. We report here a new method for quantitative analysis of sterigmatocystin using reverse phase high performance liquid chromatography.

MATERIAL AND METHODS

Fungal strains

A.versicolor and A.nidulans, isolated from various foodstuffs, were kindly supplied by Dr. Lafont (INSERM, Le Vesinet, France). The identity and purity of each strain were checked by microscopic examination.

The six Aspergillus strains were cultured in 300 ml Erlenmeyer flasks in 50 ml of Rabie medium (peptone 2 g, magnesium sulfate 0.5 g, potassium hydrogen phosphate 1 g, glucose 5 g, galactose 10 g, water 1 l) at 25°C for three weeks.

Standard solutions

Standard solutions of sterigmatocystin (Sigma) were made up in chloroform (Normapur, Prolabo) at concentrations of 0.01 µg/ml, 0.1 µg/ml, 10 µg/ml and 100 µg/ml

Sample preparation

Since sterigmatocystin is insoluble in water, it is only detectable in mycelium, and not in the culture medium (18). The extraction method described by Stack and Rodricks (12) was used. The mould cultures were homogenized in a Braun MX 32 blender, and then extracted with a mixture of acetonitrile and 4% aqueous KCl (9/1) (200 ml). After filtration (Whatman No.1) the solution was extracted with hexane (2 x 50 ml) and then chloroform (50 ml). The resulting extract was put on a silica gel column (G 60 Merck, 70-230 mesh), and the toxin was eluted with 200 ml of cyclohexane/ethyl acetate (4/1). The eluate was evaporated to dryness under nitrogen, and the residue was taken up in 1 ml of chloroform.

Identification and confirmation by Thin Layer Chromatography

20 µl of each mould extract and 10 µl of standard (10 µg/ml) were deposited on a silica plate (Merck 5553, 20x20 cm) which had been activated by heating at 110°C for 1 h and cooling in a dessicator. The plates were developed in benzene / acetic acid (90/10).

The natural red fluorescence of sterigmatocystin could be directly observed under UV illumination (254 nm, Ultra-Violet Products Inc. San Gabriel USA). Confirmation was based on transformation of the red fluorescence to bright yellow fluorescence by spraying the plates with an alcoholic solution (20%) of aluminium chloride (Aldrich S.A.), followed by heating at 80°C for 10 min. The Rf of sterigmatocystin was observed between 0.75 and 0.81 (19).

All the solvents used for the extraction and purification procedure were Normapur grade (Prolabo).

Hight Pressure Liquid Chromatography

The operating conditions were as follows: pump (Chromatem M 380 Altex-Beckman); column, μ-Bondapak C18 (mm 250 x 4, 10 μm); solvent, acetonitrile/water/acetic acid (55/45/2); flow rate, 1.5 ml/min; detector U.V. Spectromonitor 3000 (LDC Milton Roy), 320 nm (choosen after spectrophotometric study) with a 2 μl internal cell; a 10 μl injection loop was used; ambient temperature. Solvents (Normapur, Prolabo) were degassed and passed through a Millipore filter before use.

RESULTS and DISCUSSION

Under the conditions described above, sterigmatocystin was well separated with a retention time of 9.11 min (cf fig.2A). Retention times were always within +/- 10%.

A typical chromatogram of a fungal sample is shown in figure 2B. It can be seen that there were no interfering peaks. Sterigmatocystin in the samples was identified by comparison of its retention time with that of the sterigmatocystin standard. Both were around 9.05 min, with a small day to day variation due to slight differences in composition of the mobile phase.

Standard curves were constructed by plotting absorbance, expressed in units of height, versus μg of sterigmatocystin injected into the column, for five different concentrations ran-

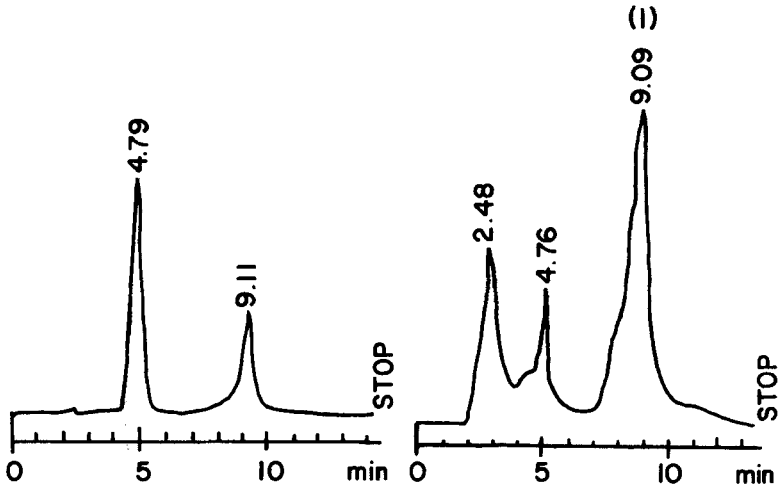

Figure 2A

Figure 2B

A) Chromatogram of sterigmatocystin standard (1 µg/ml ; sensitivity : 0.01 full scale).

B) Chromatogram of fungal strain extract (strain 1842 ; sensitivity : 0.1 full scale).

(1) ◘ sterigmatocystin (9.09 min). Column, : µ-Bondapak C18 (cm 25 x 0.46, 10 µm) at ambiant temperature. Mobile phase: acetonitrile/water/acetic-acid (55/45/2), at 1.5 ml/mn flow rate. Detector : UV absorption at 320 nm.

ging from 0.001 to 100 µg/ml at three different U.V. detector sensitivities (0.1, 0.01, 0.001). For each sensitivity, straight lines were obtained obeying equations:

Sensitivity 0.1 y = 14,400 x - 100 (r = 0.895)

Sensitivity 0.01 y ◘ 147,200 x - 500 (r = 0.947)

Sensitivity 0.001 y = 1,474,400 x - 20,000 (r ◘ 0.895)

where y is in integration units, and x is amount of sterigmatocystin injected (µg).

Recovery studies were carried out by determining sterigmatocystin concentrations in mould samples with and without addition of 1 mg of sterigmatocystin standard to the samples during preparation. Recovery for the extraction procedure was found to be 85% and for the purification step 95% (total recovery : 80%).

Injection of 4 ng of a standard solution of sterigmatocystin was found to be the limit of detection.

Determination of sterigmatocystin levels in 6 fungal cultures was also carried out. The results are shown in Table 1. It can be seen that all the strains produced significant amounts of sterigmatocystin.

In conclusion, high pressure liquid chromatography represents a convenient method for the separation and determination of sterigmatocystin in mould cultures. Although it does require preliminary sample clean-up, this method is simpler, quicker, and more sensitive than methods based on GLC.

Table 1 : Amount of sterigmatocystin in fungal strain extract (mg/g of dry mycelium).

STRAINS (and origin)	Sterigmatocystin production µg/g of mycelium
A. versicolor 1354 Cheese	0
A. versicolor 1842 Cheese	630
A. versicolor 10 Cheese	610
A. versicolor 1B Cheese	1130
A. nidulans AN3 Groundnuts	1220
A. nidulans JJ5 Groundnuts	1500

The method would be suitable for analysis of agricultural products infected by toxigenic strains of Aspergillus. From the point of view of public health and veterinary toxicology, this method of determination of sterigmatocystin using reversed phase HPLC can be seen to be an improvement on that described by Lepom (17). It is both simpler to carry out and more sensitive, and could thus be used for routine analyses.

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