Rapid and sensitive detection of citrinin production during fungal fermentation using high-performance liquid chromatography

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ABSTRACT

A rapid and sensitive assay was developed for the detection of the mycotoxin citrinin by reversed-phase chromatography. Citrinin was eluted from a radial-compression C_{18} column with a retention time of 3.86 min (flow-rate of 2.5 ml/min) with acetonitrile–water–acetic acid (40:59:1) containing tetrabutylammonium phosphate (0.025 M). Comparative analysis revealed fluorescence detection to be 100 times more sensitive than detection by conventional ultraviolet absorbance. The fluorescence excitation and emission maxima of citrinin were 330 and 500 nm, respectively. The assay was linear over the concentration range between 0.01–100 µg/ml. Recovery experiments conducted by addition of citrinin to fermentation samples, revealed the assay quantitation efficiency to be 91–102%. Assay utility was demonstrated by using an *Aspergillus niveus* culture, propagated in complex liquid medium. Citrinin production was detected as early as 20 h following inoculation and increased dramatically when the culture entered the stationary phase of growth, analogous to other secondary metabolites. Unlike previously reported methods, this procedure has the advantage of enabling the direct quantitative analysis of citrinin in crude microbial fermentations without sample extraction.

INTRODUCTION

Citrinin is a mycotoxin, produced by several species of the genera *Aspergillus* and *Penicillium* [1,2]. This secondary metabolite has been characterized as a pentaketide, synthesized from acetyl- and malonyl-coenzyme A [3,4]. Although acetyl- and malonyl-coenzyme A are commonly associated with fatty acid synthesis, these primary metabolic precursors can be utilized in a polycondensation reaction to form polyketide rings which are modified to yield a variety of secondary metabolites [3,4]. Early investigations with citrinin revealed that this compound is toxic to bacteria, fungi and protozoa [3–8]. Despite the apparent broad antimicrobial activity, therapeutic applications involving citrinin are not possibile, since citrinin has been shown to cause acute tubular necrosis in kidney [9] and impaired liver function [10].

The formation of citrinin by various fungi that contaminate feed grains has been implicated as a causative agent in renal disease and death of swine, poultry, and possibly humans [2]. An additional potential hazard may also involve therapeutics

derived from fungal fermantations. Propagation of fungi in various complex liquid media has been reported to elicit citrinin synthesis [11,12]. Consequently, citrinin analysis of fungal fermentations, utilized for the synthesis of therapeutic drugs, is imperative and requires the development of sensitive assay methodology. This paper describes a rapid reversed-phase high-performance liquid chromatography (HPLC) assay. This assay allows the highly sensitive fluorescence detection of citrinin in crude microbial fermentation samples without the need for sample extraction.

EXPERIMENTAL

Materials
        Acetonitrile and methanol (HPLC grade) and reagent-grade acetic acid were purchased from Fisher Scientific (Springfield, NJ, U.S.A.); tetrabutylammonium phosphate was from Waters (Milford, MA, U.S.A.); citrinin, $MgSO_4 \cdot 7H_2O$, $ZnSO_4 \cdot 7H_2O$, KH_2PO_4, L-lysine and glycerol were from Sigma (St. Louis, MO, U.S.A.); cerelose (90% D-glucose, 10% H_2O) was from Corn Products (Englewood Cliffs, NJ, U.S.A.); tastone-154 (yeast extract) from Universal Food Corp. (Milwaukee, WI, U.S.A.); and Bacto-agar from Difco Labs. (Detroit, MI, U.S.A.).

Culture growth conditions
        Aspergillus niveus strain ATCC 56745 was obtained form the American Type Culture Collection (Rockville, MD, U.S.A.). *A. niveus*, a known producer of citrinin [13], was propagated by inoculating conidia onto agar slants of complex medium, containing 2.0% cerelose, 1% tastone-154, 0.25% KH_2PO_4, 0.1% L-lysine, 0.025% $MgSO_4 \cdot 7H_2O$, 0.005% $ZnSO_4 \cdot 7H_2O$, 0.5% glycerol and 2% agar. The pH of the medium was adjusted to 5.2 with 1 M HCl prior to heat sterilization. Culture slants for the generation of conidia were incubated at 34°C. Liquid fermentation cultures were grown by inoculating 100 ml (300-ml erlenmeyer flask) of complex medium with conidia ($1 \cdot 10^6$/ml), harvested from 7–10-day-old culture slants. Cultures were grown at 30°C with an agitation rate of 250 rpm (G-52 shaker, New Brunswick Scientific, Edison, NJ, U.S.A.).

HPLC assay
        HPLC analysis of citrinin was performed utilizing the following Waters HPLC equipment: Model 600E pump, Model 710B WISP, Model 481 ultraviolet detector, Model 990 photodiode-array detector, Model 470 scanning fluorescence detector (16-μl flow-cell), and a Model 745 integrator. Isolation of citrinin was achieved on a Waters Radial-Pak 4-μm C_{18} analytical column (diameter, 8 mm), with a C_{18} μBondapak precolumn, inserted in-line upstream of the column. The injection volume was 10 μl for all experiments. Only deionized distilled water was used for the preparation of mobile phase solutions. The mobile phase contained acetonitrile–water–acetic acid (40:59:1) and tetrabutylammonium phosphate (0.025 M), which was filtered through a 0.45-μm hydrophobic membrane (Millipore, Milford, MA, U.S.A.) prior to use. The eluent was degassed during chromatography by constant sparging with helium (5 ml/min). The flow-rate was 2.5 ml/min.
        An ultraviolet absorbance scan of citrinin was obtained with the photodiode-array detector at 0.08 absorbance units full scale (a.u.f.s.). Analysis by ultraviolet

absorbance was performed at 330 nm at 0.001 a.u.f.s. Fluorescence scanning of both the excitation and emission spectra of citrinin was performed at medium detector sensitivity. Analysis by fluorescence detection was performed at full detector sensitivity (emission band width, 18 nm) with an excitation wavelength of 330 nm and an emission wavelength of 500 nm.

Sample preparation

Citrinin standards were prepared in methanol and stored at $-20°C$. Fermentation samples were diluted in methanol (1:1), mixed vigorously, followed by centrifugation at 2400 g for 10 min. Supernatant was removed for subsequent citrinin analysis by HPLC. Assay recovery efficiency was subsequently determined by measuring fermentation samples with known levels of exogenously added citrinin.

Dry cell weight determination

Fermentation samples (10 ml) were filtered through Whatman (Maidstone, U.K.) GF/A filter paper and washed with deionized distilled water (30 ml). The filtered mycelium was allowed to dry at room temperature for 16 h. Mycelium dry cell weight was determined using an automated volatility computer (CEM Corp., Indian Trail, NC, U.S.A.).

RESULTS AND DISCUSSION

Fermentation samples often contain a complex array of compounds of small and large molecular weight, making the accurate quantitation of of specific metabolic products difficult. Although detection of citrinin in complex samples can be enhanced by employing various extraction procedures [14–18] this approach is time-consuming, and losses may adversely influence accurate quantitation. Direct sample quantitation would require that an assay method possess a significant degree of chromatographic resolution in combination with sensitive differential detection. In this study, reversed-phase chromatography was utilized to establish an accurate and efficient assay for the direct quantitation of citrinin from fermentation samples.

Citrinin was eluted from a C_{18} (4-μm particle) radial-compression column at a flow-rate of 2.5 ml/min with a mobile phase composed of acetonitrile–water–acetic acid (40:59:1) at pH 3.8, containing the ion-pairing agent tetrabutylammonium phosphate (0.025 M). Tailing of the citrinin peak due to ionization was prevented by lowering the eluent pH with acetic acid. A guard column (C_{18} μBondapak), employed as a precolumn filter, significantly increased the analytical column life and did not adversely influence the retention time for citrinin elution.

The detection sensitivity for citrinin by ultraviolet absorbance and fluorescence spectroscopy was investigated. Fig. 1 illustrates the ultraviolet absorbance spectrum of citrinin. The ultraviolet absorbance maximum for citrinin was 330 nm. The assay was linear over the concentration range of 1–100 μg/ml. This represents a detection sensitivity similar to that previously reported by Phillips *et al.* [19]. Alternatively, various investigators have employed fluorescence detection for the analysis of citrinin [17,18,20]. Using a scanning fluorescence detector the excitation and emission spectra of citrinin were determined. As depicted in Fig. 2, the emission maximum of citrinin was 500 nm, whereas the excitation maximum was 330 nm for an emission wave-
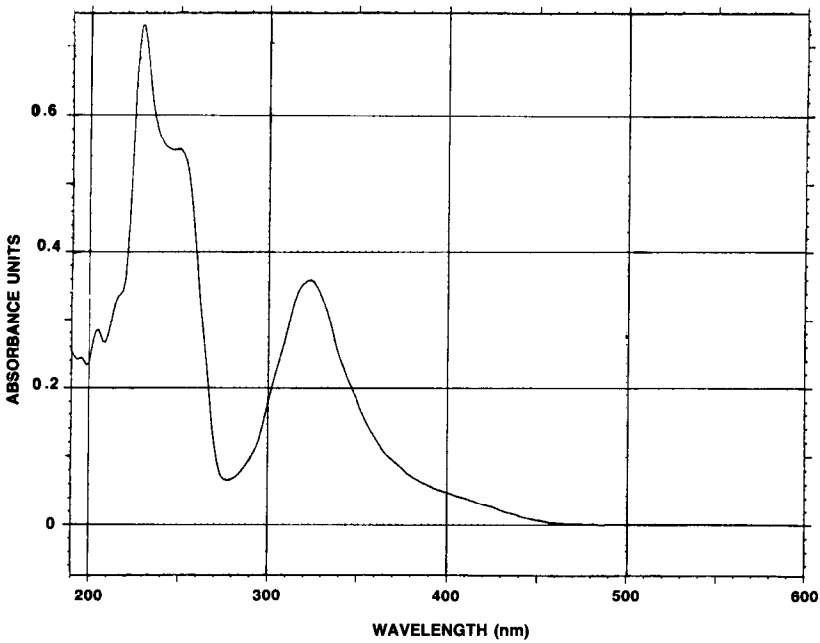
Fig. 1. UV absorbance of citrinin standard in methanol (500 μg/ml).

length of 500 nm. The assay was linear over the concentration range 0.01–100 μg/ml. As little as 0.1 ng of citrinin in a 10-μl injection volume could be accurately measured by fluorescence detection. Only 10 ng of citrinin (10-μl injection volume) could be accurately measured by ultraviolet absorbance detection. This indicates that fluorescence detection is approximately 100 times more sensitive than ultraviolet absorbance. Levels of citrinin in fermentation samples that were undetectable by ultraviolet absorbance, were accurately quantified using fluorescence detection (Fig. 3).

    Assay reproducibility with fluorescence detection was evaluated by 30 succes-

TABLE I

ANALYSIS OF ASSAY REPRODUCIBILITY FOR CITRININ BY HPLC WITH FLUORESCENCE DETECTION

Thirty successive 10-μl injections, each of 2.5 or 100 ng per injection, of citrinin were performed. The peak height and area reported have been normalized to 1 ng per injection. The separation of citrinin was carried out at full detector sensitivity, as described in Experimental.

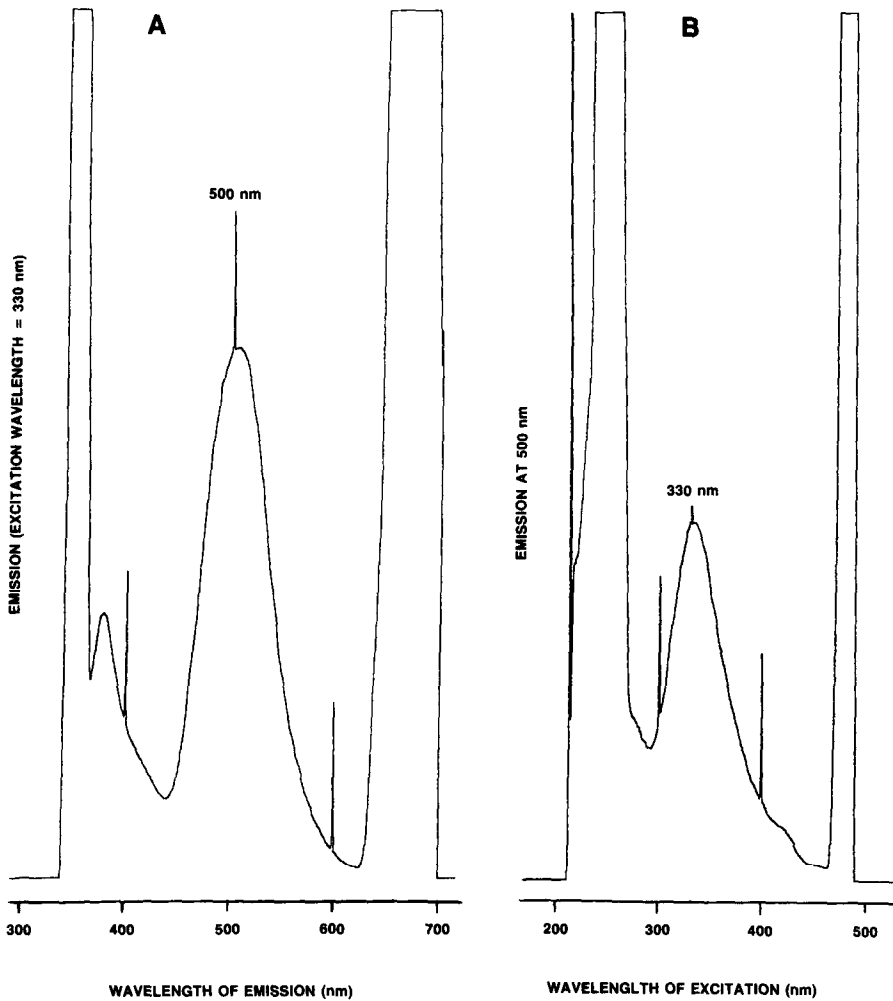|	Peak area (μV · s)	Peak height (μV)	Retention time (s)
Range (× 1000)	320–351	9.9–11.1	228.0–233.4
Mean (× 1000)	335	10.4	231.6
Standard deviation (× 1000)	9.0	0.25	1.6
Coefficient of variation (%)	2.7	2.4	0.7
Sensitivity (× 1000)	335	10.4	

Fig. 2. Fluorescence scan of the emission and excitation spectra of citrinin. (A) Emission scan of citrinin in methanol (10 μg/ml), at the UV absorbance maximum of 330 nm as the excitation wavelength. (B) Excitation scan of citrinin in methanol (10 μg/ml) at the emission maximum of 500 nm observed in (A).

TABLE II

RECOVERY OF CITRININ FROM FERMENTATION SAMPLES

Known amounts of citrinin were added to a 20-h culture of *A. niveus* (ATCC 56745), grown in complex medium, and subsquently analyzed for citrinin by fluorescence detection, as described in Experimental. Values represent an average of two duplicate samples, each assayed in triplicate.

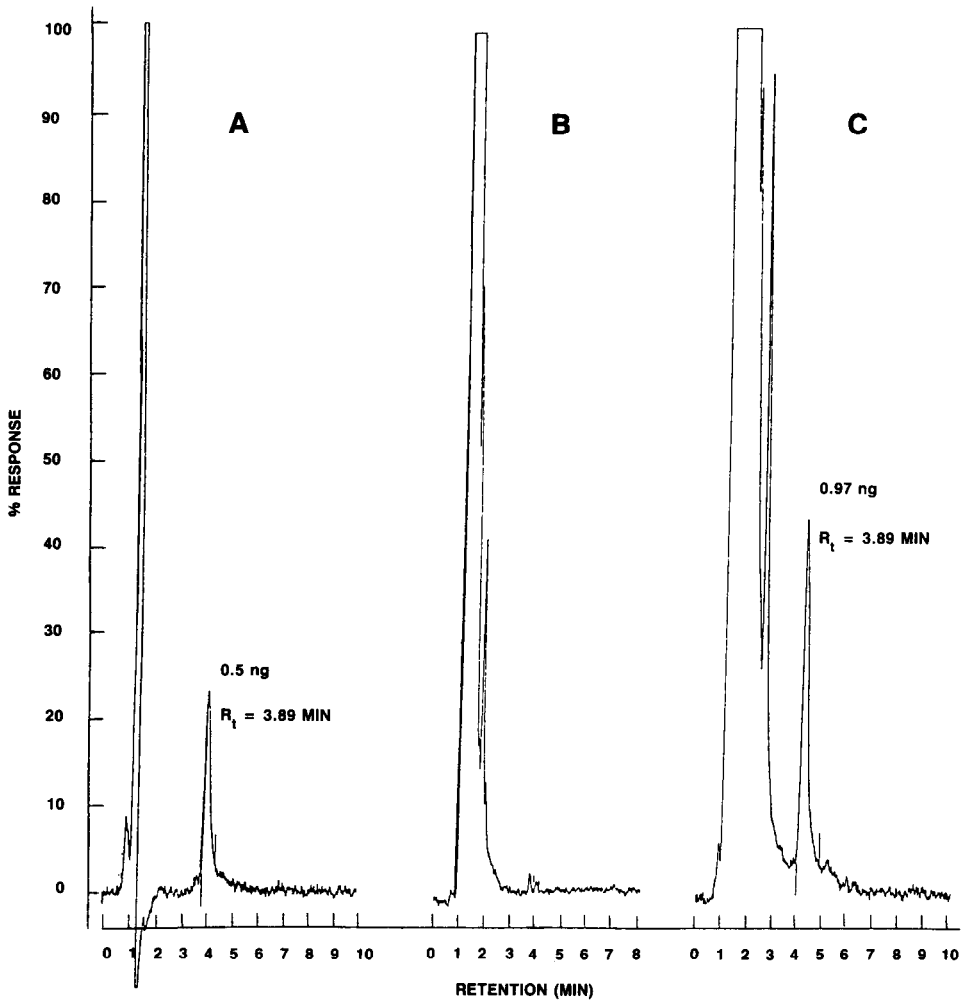| Citrinin added (μg/ml)	Amount detected (μg/ml)	Recovery (%)	Standard deviation
0.0	0.00	—	—
0.1	0.10	102	5.3
2.0	1.82	91	0.7
5.0	4.88	97	3.8
10.0	10.00	100	2.0

Fig. 3. Chromatograms of *A. niveus* fermentation sample. (A) Citrinin standard in methanol (50 ng/ml) using fluorescence detection. (B) UV detection of a 20-h *A. niveus* fermentation sample. (C) Fluorescence detection of the identical fermentation sample depicted in (B). R_t = Retention time.

sive injections (10 μl) each of 2.5 ng or 100 ng of citrinin standard. Table I lists the data obtained for the measurement of peak height, peak area and retention time. Citrinin quantitation was evaluated by adding known amounts of citrinin to fermentation samples prior to analysis. Quantitation efficiency of citrinin in fermentation samples was 91–102% (Table II). Similar efficiencies for the analysis of citrinin from plasma, urine and bile samples were previously reported by Phillips *et al.* [19]. For ultraviolet absorbance, the detection sensitivity limit of this method was only 10–30 ng. Accurate quantitation of citrinin was achieved without extensive sample extraction protocols or pre-column concentration [17,20].

This methodology was applied to a study of citrinin production by an *Aspergil-*

lus niveus culture (ATCC 56745), propagated in complex liquid medium. Trace levels of citrinin production were detected as early as 20 h following inoculation. Citrinin synthesis increased dramatically when the culture entered the stationary phase of growth, yielding a concentration of 270 μg/ml following a 72 h fermentation. This result is in agreement with earlier studies indicating that citrinin biosynthesis by *A. niveus* is associated with secondary metabolism [3,4]. Characterization of the factors involved in the regulation of citrinin synthesis will require further investigation.

ACKNOWLEDGEMENTS

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