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Note

Simultaneous determination of the mycotoxins citrinin and ochratoxin A in wheat and barley by high-performance liquid chromatography

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Ochratoxin A and citrinin are nephrotoxic secondary metabolites produced by several fungal species of the genuses *Penicillium* and *Aspergillus*[1–3]. Although the toxins have been detected in a number of agricultural commodities at geographically diverse locations, the principal interest has involved studies of cereal feed grains in Scandinavia[2,4]. Mycotoxic porcine nephropathy, a disease occurring endemically in Denmark and some other countries[2,4–6], has been suggested as being caused by ingestion of mouldy feed contaminated with ochratoxin- and citrinin-producing strains of *Penicillium viridicatum*[2,5]. For this reason, it seemed to be desirable to develop a reliable method for the determination of these two mycotoxins in cereals.

Several multi-mycotoxin detection methods, including the simultaneous determination of ochratoxin A and citrinin using thin-layer chromatography (TLC), have been described[7–12], but only incomplete data on method sensitivity and recoveries are available. Low sensitivities and poor recoveries, especially for citrinin, were reported and therefore the methods are suitable for qualitative or screening purposes at best. On the other hand, high-performance liquid chromatography (HPLC) has been applied to the individual analyses of ochratoxin A[13–20] and citrinin[21,22] in cereals at ppb* levels and in one instance to their simultaneous determination in cheese[23]. The purpose of this work was to develop a simple method allowing the simultaneous extraction, purification and detection of ochratoxin A and citrinin that is economical in labour and both sensitive and quantitative.

This paper describes a modification of the method of Marti *et al.*[21] for the determination of citrinin in corn and barley to quantitate citrinin and ochratoxin A simultaneously using a unique extraction and detection procedure.

EXPERIMENTAL

Chemicals

Ochratoxin A was purchased from Sigma (St. Louis, MO, U.S.A.). A stock solution (120 μg/ml) was prepared in benzene–acetic acid (99:1). Standard citrinin was prepared in our laboratory according to a literature method[24]. Its purity was

* The American billion (10^9) is meant throughout the article.

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confirmed by TLC, HPLC and UV spectrometry. A stock solution was prepared by dissolving 3 mg of citrinin in 100 ml methanol. For HPLC, the stock solution was evaporated to dryness and dissolved in the HPLC mobile phase. All solvents were distilled before use.

Apparatus

HPLC was performed using a Hewlett-Packard Model 1010B liquid chromatograph equipped with an LDC Model 1209 fluorescence detector (excitation wavelength 360 nm). Separations were achieved with a stainless-steel column (25 × 0.4 cm I.D.) containing Separon SI C_{18} (particle size 10 μm) (Laboratorni Přistroje, Prague, Czechoslovakia) at an elution rate of 1.5 ml/min at 40°C. Samples were injected on to the column by means of a six-port loop injector (Rheodyne Model 7010). An HP 3380A plotting integrator was used to calculate peak areas.

Eluent

The eluent was acetonitrile–isopropanol–$8 \cdot 10^{-2}$ *M* phosphoric acid (35:10:55). The eluent was filtered and degassed before use.

Extraction and separation

A 25-g sample was extracted with 200 ml of acetonitrile–water (9:1), the latter containing 4% of potassium chloride and 0.4 ml of concentrated sulphuric acid, using a wrist-action shaker. After filtration, a 100-ml aliquot was defatted twice with 50 ml of *n*-hexane. After adding 25 ml of water, the acetonitrile–water phase was extracted three times with chloroform (50, 10 and 10 ml). The combined chloroform layers were partitioned three times against 25 ml of 5% sodium hydrogen carbonate solution. The combined aqueous phases were acidified to pH 1.5 by adding concentrated hydrochloric acid and re-extracted with three portions of chloroform (50, 25 and 25 ml). The chloroform layers were collected over anhydrous sodium sulphate, filtered and rotary evaporated at room temperature. The residue was transferred quantitatively into a 10-ml flask with sequential portions of chloroform (3 × 1 ml) and the solvent was concentrated to dryness under a stream of nitrogen. The residue was dissolved in an appropriate volume of mobile phase (1–5 ml).

Aliquots of 20 μl were injected on to the HPLC column. Calibration graphs for citrinin and ochratoxin A in the concentration ranges 2.5–25 and 10–70 ng, respectively, were monitored daily. Detection was performed by fluorescence measurement at full detector sensitivity.

RESULTS AND DISCUSSION

Ochratoxin A and citrinin possess acidic properties, permitting their simultaneous extraction with the same solvent and purification by acid–base partition. The clean-up procedure described is based on the method of Marti *et al.*[21] for the determination of citrinin in corn and barley. It is possible to reduce the sample size and the volumes of the solvents to make the procedure more economic and less time consuming. At present 10-g samples extracted and purified with half of the solvent volumes mentioned above are being analysed in our laboratory.

Table I gives the recovery and precision data for wheat and barley to which

TABLE I

RECOVERY OF CITRININ AND OCHRATOXIN A FROM WHEAT AND BARLEY

Averages of four consecutive analyses.

Substrate	*Added (ng/g)*	*Citrinin*			*Ochratoxin A*		
		Found (ng/g)	*C.V.* (ng/g)*	*Recovery (%)*	*Found (%)*	*C.V.* (ng/g)*	*Recovery (%)*
Wheat	100	71.3	3.7	71.3	86.1	7.5	86.1
	500	n.a.**			419.5	4.9	83.9
	1000	695.2	4.4	69.5	n.a.		
Barley	100	77.0	8.2	77.0	89.4	8.7	89.4
	500	n.a.			428.7	5.5	85.7
	1000	625.0	5.2	62.5	n.a.		
Mean			5.4	70.1		6.6	86.3

* Coefficient of variation.
** Not analysed.

ochratoxin A and citrinin had been added. The results obtained demonstrate the high reproducibility of the method, although the recoveries for citrinin from grain are not entirely satisfactory. Extraction of citrinin from feedstuffs is a difficult problem that is matrix-dependent and unsolved so far. Addition of chelating agents such as salts of EDTA to the solvent to improve the extraction of citrinin as proposed by Wilson and co-workers[25,26] was found to be ineffective. Another factor that strongly influences the recovery is the effect of pH on the properties of citrinin. Possibly citrinin forms irreversibly insoluble salts during treatment with sodium hydrogen carbonate solution that prevent complete re-extraction with chloroform after acidification.

Atttempts to replace acid–base partition by column chromatography for sample clean-up to improve the recovery of citrinin have failed to give positive results.

The procedure is also suitable for the determination of ochratoxin A and citrinin in corn, but according to Wilson[25] citrinin is difficdult to quantitate in this substrate because of the variable recoveries obtained.

The HPLC separation of ochratoxin A and citrinin was achieved using a Separon SI C_{18} (particle size 10 μm) column with acetonitrile–8 · 10^{-2} *M* phosphoric acid (50:50) or acetonitrile–isopropanol–8 · 10^{-2} *M* phosphoric acid (35:10:55), previously reported for the separation of citrinin in biological fluids[22].

It is necessary to use an acidic eluent to suppress the ionization of the two mycotoxins and peak tailing. Under these conditions citrinin and ochratoxin A were well separated from one another and eluted as sharp peaks within 7 min. No interfering peaks were observed in the chromatograms of wheat and barley samples (Fig. 1) and sensitive quantitation of the toxins is possible. A decrease in detector sensitivity occurred when methanol was used instead of acetonitrile in the mobile phase.

Seventeen injections of citrinin and ochratoxin A standard solutions gave mean retention times of 268 and 396 s with coefficients of variation of 0.56 and 1.05%, respectively (Table II). Despite the use of an acidic eluent, no decrease in column efficiency was observed over an 8-week period of daily injections. The relationships

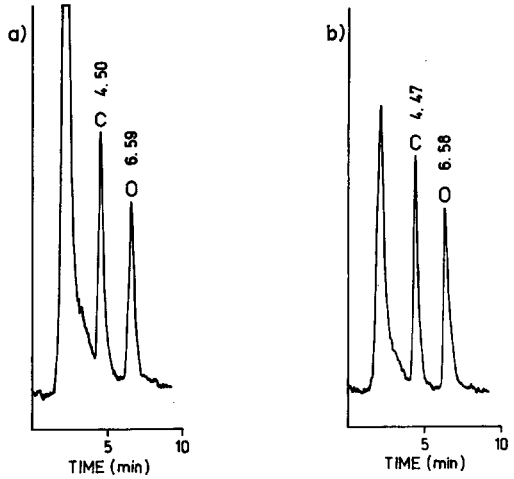

Fig. 1. HPLC separation of citrinin and ochratoxin A on a Separon SI C_{18} column with acetonitrile–isopropanol–8 · 10^{-2} *M* phosphoric acid (35:10:55) as eluent and fluorescence detection. (a) Barley sample spiked with 100 ng/g of citrinin (C) and 240 ng/g of ochratoxin A (O); (b) wheat sample spiked with 100 ng/g of citrinin (C) and 240 ng/g of ochratoxin A (O).

TABLE II

REPRODUCIBILITY OF RETENTION TIMES FOR CITRININ AND OCHRATOXIN A

Parameter	*Citrinin*	*Ochratoxin A*
No. of injections	17	17
Retention time:		
Range (s)	266–272	392–398
Mean (s)	268	396
Standard deviation (s)	1.5	4.2
Coefficient of variation (%)	0.56	1.05

between peak height and area and the amount injected were linear over the ranges 2.5–50 and 10–100 ng for citrinin and ochratoxin A, respectively.

The detection limits were 10 ng/g for citrinin and 40 ng/g for ochratoxin A. A considerable increase in sensitivity for ochratoxin A can be achieved by using an excitation wavelength of 335 nm for fluorescence detection, but a detector with a variable excitation wavelength was not available. The analysis time is less than 10 min.

The HPLC procedure described here for the determination of citrinin and ochratoxin A in cereals offers several advantages over previously reported TLC methods[7–12] with respect to sensitivity, recovery, analysis time and quantitation. It allows the sensitive analysis of these two mycotoxins in grain extracts after a simple acid–base partition clean-up and is well suited to their simultaneous monitoring in suspect feed samples.

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