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An easy screening method for fungi producing ochratoxin A in pure culture

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Abstract

A simple screening method has been developed for detecting ochratoxin production by fungi, based on high-performance liquid chromatographic determinations on extracts obtained from agar plugs cut from pure Petri dish cultures. Two culture media, Yeast Extract Sucrose agar and Czapek Yeast Extract agar, and three extraction solvents (methanol, methylene chloride/formic acid, and methanol/formic acid) were compared. All of the isolates tested produced ochratoxin A in one or both culture media after 7 or 14 days of incubation. Based on the results obtained, the use of both culture media is recommended. As extraction solvent, either methanol or methanol-formic acid could be used. This method also provides quantitative information on the level of ochratoxin produced by the cultures. The simplicity of the method makes it very useful when many fungal isolates need to be screened. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Ochratoxin A; Fungi; Mycotoxins

1. Introduction

Ochratoxins comprise the first major group of mycotoxins identified after the discovery of the aflatoxins. Ochratoxin A (OA) is the most toxic compound of this group, and therefore, it is receiving increasing attention for its nephrotoxic effects and its potential carcinogenic activity (Kuiper-Goodman and Scott, 1989; Pohland, 1993).

Numerous analytical methods have been described to detect toxigenic fungi based on toxin production in natural or synthetic substrates followed by extraction, purification and detection by thin layer chromatography (TLC) or high-performance liquid chromatography (HPLC). In general, these methods are time-consuming and are not suitable when many fungal isolates need to be screened. Some simpler techniques were developed, particularly the agar plug method (Filtenborg and Frisvad, 1980; Filtenborg et al., 1983). This method involves skillful manipulation of agar plugs onto the thin layer plate surface and can be tedious, especially if more than one plug needs to be superimposed. In order to obtain a full profile of secondary metabolites, Smedsgaard (1997) reported a method based on the extraction of agar plugs from *Penicillium* cultures. Using HPLC with

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Table 1
Aspergillus strains used in this study

Species	Strain (source)
A. ochraceus	A-8 (feedstuffs, CCFVB)
A. ochraceus	A-210 (feedstuffs, CCFVB)
A. alliaceus	A-196 (feedstuffs, CCFVB)
A. sclerotiorum	FRR 4491
A. awamori	NRRL 3112
A. foetidus	CBS 618.78
A. niger var. *niger*	A-75 (feedstuffs, CCFVB)
A. niger var. *niger*	A-136 (soy, CCFVB)
A. carbonarius	N-1 (supplied by Dr. F. Kevei and J. Varga)
A. carbonarius	N-2 (supplied by Dr. F. Kevei and J. Varga)
A. carbonarius	1.4.29 (soil, supplied by Dr. F. Kevei and J. Varga)
A. carbonarius	IMI 041875
A. carbonarius	NRRL 67

Abbreviations: FRR, Food Science Australia, North Ryde, NSW, Australia; NRRL, Northern Agricultural Research Service Culture Collection, Peoria, IL, USA; CBS, Centraalbureau voor Schimmelcultures, Baarn, Netherlands; IMI, International Mycological Institute, Surrey, Egham, UK; CCFVB, Culture Collection of Veterinary Faculty of Barcelona, Spain.

diode array detection, the chromatographic metabolite profiles obtained were very useful in studies of fungal chemotaxonomy.

The purpose of this study was to develop a simple and clean method based on HPLC detection and quantification of OA in the extract obtained from agar plugs, enabling mycotoxin production to be detected in a small sample.

2. Materials and methods

2.1. Culture media

The culture media used were Yeast Extract Sucrose (YES) agar, which contained per litre: yeast extract, 20 g; sucrose, 150 g; agar, 20 g and magnesium sulfate, 0.5 g. Czapek Yeast Extract (CYA) agar, which contained per litre: K_2HPO_4, 1 g; Czapek concentrate, 10 ml; trace metal solution, 1 ml; yeast extract, 5 g; sucrose, 30 g; agar, 15 g (Pitt and Hocking, 1997).

2.2. Recovery studies

For recovery studies, 5 ml of melted agar (YES and CYA) were added to 55-mm Petri dishes. Before the agar solidified, 100 μl of chloroform containing 34.3, 17.1 and 7.7 mg of OA standard (Sigma, St. Louis, MO) were added aseptically to the Petri dishes to give 6.9, 3.4 and 1.5 μg of OA per ml of culture media, respectively. Three agar plugs were removed from each medium and mycotoxin concentration, weighed and introduced into a small vial. Three different extraction solvents were evaluated: methanol, methylene chloride/formic acid (25:1) (Abramson and Clear, 1996) and methanol/formic acid (25:1). A volume of 0.5 ml of each solvent was added. After 60 min, the extracts were filtered (Millex® HV 13 mm, Millipore) and injected into the HPLC as previously described (Abarca et al., 1994). The HPLC apparatus used was a model 500B-G (Konik, Barcelona, Spain) with a loop of 20 μl and equipped with a fluorescence detector (Konik, model

Table 2
Recovery of added ochratoxin A from two media with three solvent systems[a]

Ochratoxin A added to 1 ml of media (μg)	Recovery (%)					
	Methanol		Methylene chloride/formic acid (25:1)		Methanol/formic acid (25:1)	
	YES	CYA	YES	CYA	YES	CYA
1.5	53.7	96.1	67.2	106.4	101.9	112.4
3.4	42.1	81.3	58.3	89.9	78.1	104.4
6.9	34.9	85.9	51.8	87.0	69.2	65.7

[a]YES, yeast extract sucrose agar; CYA, Czapek yeast extract agar.

Table 3
Concentration of ochratoxin A (μg/g) produced by several *Aspergillus* species in two media after 7, 14 and 21 days of incubation at 25 °C, using two extraction solvents[a]

Species (reference)	YES						CYA					
	Methanol			Methanol/formic acid (25:1)			Methanol			Methanol/formic acid (25:1)		
	7 days	14 days	21 days	7 days	14 days	21 days	7 days	14 days	21 days	7 days	14 days	21 days
A. ochraceus (A-8)	49.0	36.2	29.7	52.6	38.0	26.3	ND	ND	ND	ND	ND	ND
A. ochraceus (A-210)	36.3	2.4	4.0	38.1	7.2	3.9	ND	ND	ND	ND	ND	ND
A. alliaceus	1.3	91.1	51.8	1.6	91.2	52.2	0.8	15.5	29.4	1.0	15.2	30.3
A. sclerotiorum	ND	ND	5.4	ND	ND	3.4	1.9	3.7	3.3	1.5	3.1	3.4
A. awamori	43.5	27.0	11.7	35.5	28.9	15.5	30.2	36.0	37.1	50.4	28.2	39.1
A. foetidus	64.0	9.9	5.9	58.9	13.3	9.7	94.8	54.1	80.6	92.7	64.7	71.8
A. niger var. *niger* (A-75)	50.7	41.9	74.3	61.1	21.5	81.5	1.6	1.3	2.4	1.4	3.5	3.1
A. niger var. *niger* (A-136)	51.7	24.7	13.2	47.6	23.0	13.6	12.3	1.6	2.2	9.8	3.2	2.0
A. carbonarius (N-1)	3.4	4.0	5.1	3.5	5.4	8.6	9.7	20.9	19.3	18.2	17.9	19.7
A. carbonarius (N-2)	1.9	4.9	3.7	4.7	2.7	6.9	13.3	27.5	21.7	12.8	24.8	22.6
A. carbonarius (1.4.29)	6.1	4.0	2.9	3.6	3.8	3.5	14.4	49.5	27.6	19.9	62.0	38.4
A. carbonarius (IMI 041875)	2.8	4.3	5.3	7.3	4.5	15.9	41.8	32.6	29.2	37.7	34.3	34.0
A. carbonarius (NRRL 67)	1.9	15.9	4.1	9.7	4.1	4.1	18.1	32.4	28.8	17.2	37.4	34.3

ND, not detected.
Detection limit for ochratoxin A, 0.18 μg/g.
[a]YES, yeast extract sucrose agar; CYA, Czapek yeast extract agar.

403) (excitation 330 nm, emission 460 nm) and C18 column (Spherisorb ODS 2, 250 × 4.6 mm, 5 μm). The mobile phase was pumped at 1.0 ml min^{-1} and consisted of an isocratic program of 57% acetonitrile, 41% water and 2% acetic acid. OA was quantified on the basis of the HPLC fluorometric response compared with that of an OA standard. When methylene chloride/formic acid was used as extraction solvent, the filtered extracts were evaporated and redissolved in 0.5 ml of methanol prior to the injection.

2.3. Fungal strains

Thirteen *Aspergillus* isolates were used in the present study (Table 1), all from species which had been reported as ochratoxin producers in *Aspergillus* sections *Circumdati* (Abarca et al., 1997; Varga et al., 1996) and *Nigri* (Abarca et al., 1994; Téren et al., 1996). Five (A-8, A-75, A-136, A-196 and A-210) were isolated in our laboratory from animal mixed feeds and raw materials and the remaining strains were kindly supplied by F. Kevei and J. Varga (Attila József University, Szeged, Hungary).

2.4. Preliminary screening with a qualitative TLC method

All the isolates were three-point inoculated into YES agar and incubated at 25 °C for 7 days. Three agar plugs were removed from the central area of the colony and transferred to TLC plates using the agar plug method of Filtenborg and Frisvad (1980).

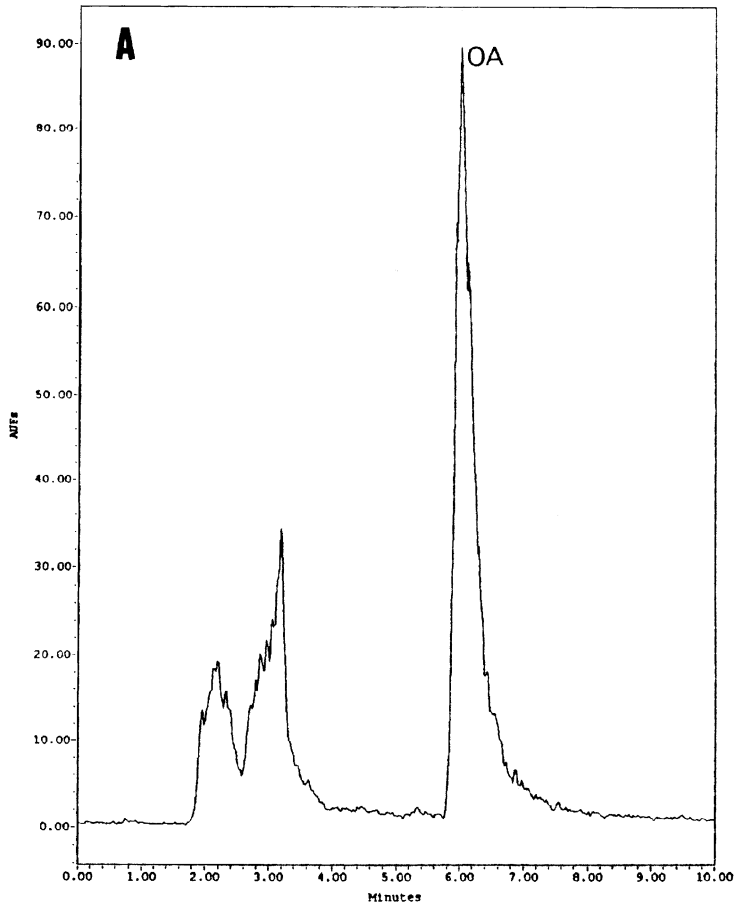

Fig. 1. HPLC chromatograms for the strain *A. niger* var. *niger* A-136 after 7 days of incubation, (A) in YES (51.7 μg/g) and (B) in CYA (12.3 μg/g), using methanol as extraction solvent. Retention time of OA, 6.5 min; AUFs, response fluorometric detector.

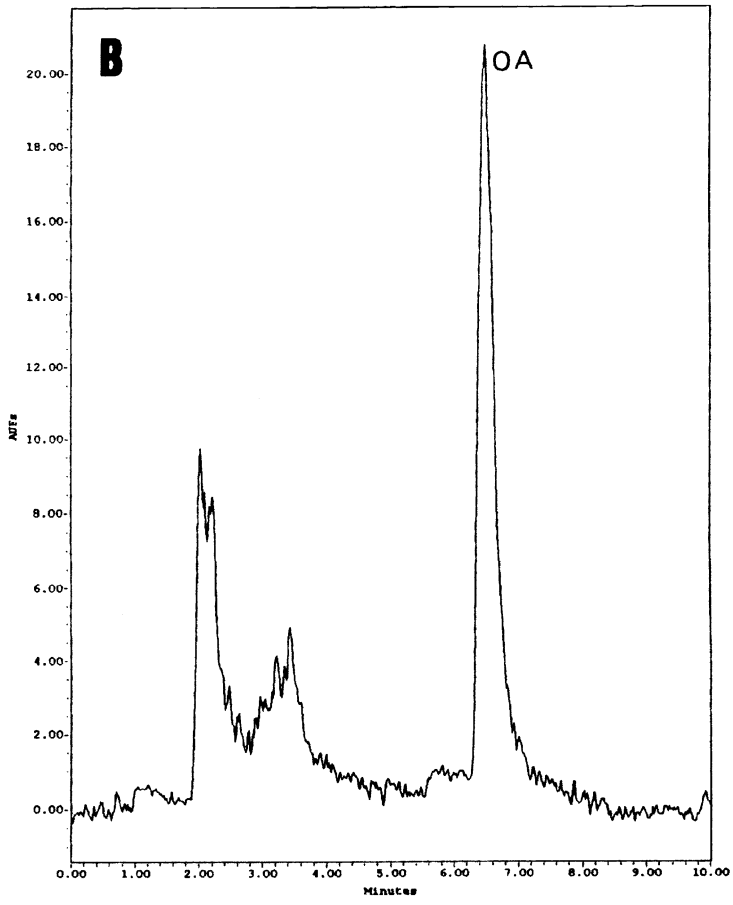

Fig. 1 (*continued*).

2.5. HPLC method

For this HPLC screening method, the strains were three-point inoculated into YES and CYA agar and analyzed after 7, 14 and 21 days growth at 25 °C. At each time, three agar plugs were removed from the central area of the colony, weighed and introduced into a small vial. A volume of 0.5 ml of methanol or methanol/formic acid (25:1) was added to each vial for 60 min and extracted and quantified as described above.

3. Results and discussion

The recovery of added ochratoxin A from YES and CYA is shown in Table 2. Generally, a higher recovery of OA was obtained from CYA than from YES agar, although no statistically significant differences were found ($p = 0.061$). Abramson and Clear (1996) reported that such large differences in recoveries from both culture media could be related to sucrose content: CYA (3% sucrose) probably presents a less hydrophilic layer more permeable to lipophilic solvents compared to YES agar (15% sucrose). As shown in Table 2, the addition of formic acid in the extraction solvent improved the recovery of the acidic OA, especially from YES agar. As no statistically significant differences were found ($p = 0.662$), we chose methanol and methanol/formic acid (25:1) as extraction solvents, to avoid the evaporation needed when methylene chloride/formic acid was used.

In preliminary screening using the agar plug TLC method, ochratoxin production was detected in all of the strains except *Aspergillus sclerotiorum* FRR 4491. Téren et al. (1996) reported that this TLC method was not sufficiently sensitive to detect OA production using the same isolates of the *Aspergillus* section *Nigri* (except A-75 isolate).

Using this HPLC method, OA production by all isolates was detected in one or both culture media (Table 3). OA was not detected in *A. sclerotiorum* after 7 days of incubation in YES medium.

The optimal medium for production of secondary metabolites by fungi depends on the metabolite, species and isolate. In the agar plug TLC method, YES agar was recommended to detect extracellular mycotoxins including OA (Frisvad and Filtenborg, 1983). The addition of $MgSO_4$ to YES increased mycotoxin production (Filtenborg et al., 1990). The use of CYA rather than YES agar in studies on citrinin and ochratoxin was recommended by Abramson and Clear (1996). In our study, statistically significant differences were not found in the OA concentration produced on either culture medium ($p = 0.822$) or detected by either extraction solvents ($p = 0.898$). YES medium was a good substrate for OA production in *Aspergillus* section *Circumdati* strains and the two *A. niger* isolates studied. For the remaining isolates, especially those of *A. carbonarius*, maximum OA production was detected after growth on CYA. Based on these results, we recommend the use of both culture media to detect the production of this mycotoxin. Either methanol or methanol/formic acid (25:1) can be used as extraction solvents. Seven days of incubation was usually sufficient to permit OA detection, but some isolates would require 14 days to reach maximum production.

The present method provides a rapid, clean way for detecting production of OA by fungi, and quantifies OA production in pure culture. It has several advantages over other conventional methods which use large amounts of natural substrates or culture media, and subsequently, are quite costly in terms of solvents and amount of work. The simplicity of the method makes it very useful when many fungal isolates need to be screened. Another advantage is that the extracts are very clean and the HPLC chromatograms show no interference (Fig. 1). The suitability of this method for other mycotoxins is under study.

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