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PROFILING OF *ALTERNARIA* MYCOTOXINS IN FOODSTUFFS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH DIODE-ARRAY ULTRAVIOLET DETECTION

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SUMMARY

The potential of high-performance liquid chromatography (HPLC) with diode-array detection for the profiling of mycotoxins in food samples has been demonstrated. A gradient elution reversed-phase chromatographic method coupled with a suitable extraction procedure was devised for the separation and detection of major *Alternaria* mycotoxins in foodstuffs. Altenuene, alternariol, alternariol methyl ether (dibenzo-α-pyrone derivatives), altertoxin-I and altertoxin-II (perylene derivatives) were profiled in extracts of artificially infected maize, rice and tomato samples and naturally contaminated sunflower seeds. First evidence of the occurrence of a new dibenzo-α-pyrone derivative in *Alternaria* cultures is also reported.

INTRODUCTION

The genus *Alternaria* is one of the most common moulds contaminating foods and feeds, causing considerable economic losses to the food industry due to rotting of fruits and vegetables[1]. *Alternaria* produces many metabolites of different chemical structure, and some exhibit mammalian toxicity. The dibenzo-α-pyrone and perylene derivatives shown in Fig. 1 are, together with tenuazonic acid, the *Alternaria* metabolites of major concern because of their toxicity and/or natural occurrence in a number of fungal-contaminated commodities.

Thin-layer chromatography is the most widely used technique for the detection of these mycotoxins (for a review, see ref. 2), although gas chromatography (with flame ionization[3] or mass spectrometric detection[4]) and high-performance liquid chromatography (HPLC)[5–11] have also been used with varying degrees of success. According to Schade and King[2], HPLC may eventually be the procedure of choice for detecting *Alternaria* mycotoxins, but further developments are necessary to ensure the identification and quantitation of some toxins in foodstuff extracts.

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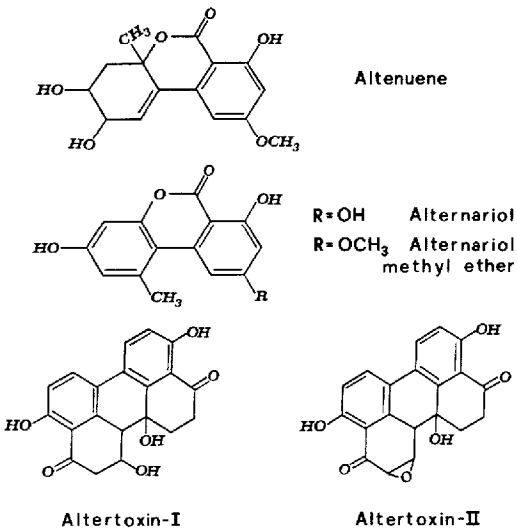

Fig. 1. Structures of representative dibenzo-α-pyrone and perylene derivatives.

Profiling of mycotoxins in fungal-contaminated foodstuffs by liquid chromatography coupled to detectors that are unable to give retention data corroborated by peak purity and peak identity tests may result in unreliability owing to the complexity of the matrix. The use of a blank extract (obtained from an uncontaminated sample) to check for potential interferents does not necessarily solve the problem. With such an approach interferences arising from fungal metabolites other than the selected toxins cannot be definitely excluded.

The advent of computer-controlled photodiode-array UV detectors in HPLC[12] has provided considerable help solving such a complex analytical problem by greatly improving peak identification, peak purity assessment and quantitation. The high spectral acquisition rate during elution provides a matrix of absorbance–wavelength–time data that can be treated by computer-aided techniques for their reduction, manipulation and presentation.

In this paper, the potential of diode-array detection in the HPLC analysis of *Alternaria* contaminated foodstuffs is demonstrated. A gradient elution reversed-phase chromatographic method was devised that simultaneously separated altenuene (ALT), alternariol (AOH), alternariol methyl ether (AME), altertoxin-I (ATX-I) and altertoxin-II (ATX-II). Tenuazonic acid, actually isolated in the extraction step, is determined separately using a procedure described elsewhere[9] and is not considered here. An *Alternaria* metabolite with spectral characteristics very similar to those of altenuene (but separately eluting) has been found in the extract of *Alternaria alternata* (Fr.) Keiss. cultured on rice.

EXPERIMENTAL

Chemicals

Reference standards of AOH, AME, ALT, ATX-I and ATX-II were produced and isolated as described elsewhere[9].

Water and methanol were of HPLC grade (Carlo Erba, Milan, Italy). All other chemicals were of analytical-reagent grade.

Apparatus

A Perkin-Elmer (Norwalk, CT, U.S.A.) Model 3B pump module equipped with a Rheodyne 7125 injection valve and a 125 × 4.6 mm I.D. reversed-phase ODS column (Bischoff, Leonberg, F.R.G.) with a Spherisorb ODS (5 μm) packing was used as the chromatographic system. A Bischoff guard column (30 × 4.6 mm I.D.) with Spherisorb ODS (5 μm) packing was used to protect the analytical column. The detector was an HP 1040A photodiode-array spectrophotometer (Hewlett-Packard, Palo Alto, CA, U.S.A.) interfaced to an HP 85 computer equipped with an HP 9121 dual disk drive and an HP 7470A plotter.

Sources of contaminated samples

Strains of *Alternaria alternata* were cultured on rice, maize and tomatoes in order to have artificially infected samples. For example, rice (200 g) was brought to about 50% moisture in a 500-ml erlenmeyer flask, autoclaved for 20 min at 120°C before fungal inoculation and then incubated in the dark for 3 weeks at room temperature (this is one of the best ways to simulate naturally contaminated foodstuffs).

Sunflower seeds naturally infected by *Alternaria alternata* were collected in the field in southern Italy and finely ground in a Buehler mill before toxin extraction.

Toxin extraction

Undried cultures or naturally contaminated samples (50 g) were extracted in a blender with 75 ml of methanol and filtered. A 40-ml portion of the filtrate was clarified by addition of 80 ml of 5% aqueous ammonium sulphate and filtered. A 90-ml volume of the filtrate (corresponding to 20 g of the original substrate) was extracted twice with 5 ml of methylene chloride. For oleaginous samples (sunflower seeds or corn kernels) a defatting step, with 30 ml of *n*-hexane, preceded the methylene chloride extraction. The combined extracts containing the dibenzo-α-pyrone and perylene derivatives were evaporated to dryness and reconstituted with 1 ml of methanol. The aqueous layer could be processed according to a procedure described elsewhere[9] to determine tenuazonic acid.

For recovery experiments the uncontaminated substrate was spiked with the required amount of standard toxins, extracted as above and analysed by HPLC. Recovery was determined by calculating the peak-height ratio of samples and standards. Each experiment was done in triplicate.

Chromatographic conditions

A binary gradient system composed of methanol (solvent A) and H_3PO_4-acidified (pH 3) water was used. The elution programme was (1) from 50 to 70% solvent

A in 10 min, (2) from 70 to 85% in 0.1 min, then (3) isocratically for 8 min. The equilibration time between two successive runs was 8 min.

The injection volume was 2 – 5 μl of the crude extract. Unless specified otherwise, spectra were acquired in the 210 – 400 nm range on the apex and on the ascending or descending part of each peak using a pilot signal at 257 nm, a reference signal at 550 nm (100 nm band width) and a threshold value of 1 mAU[13]. Up to seven chromatograms at different wavelengths could be retrieved and processed after the chromatographic run. Reference spectra were acquired during the elution of a standard mixture and used for peak identification purposes by comparison after spectral normalization.

Thin-layer chromatography was performed as described elsewhere[9].

RESULTS AND DISCUSSION

A standard mixture of ALT, AOH, AME, ATX-I and ATX-II was injected in order to obtain retention time data and an archive of reference spectra under the operational experimental conditions for the samples. Linearity of the absorbance *vs.* concentration plot was observed for more than three decades of concentration and detection limits calculated from the calibration parameters were 5, 10, 3 and 10 ng on-column for ALT, ATX-I, AOH, and AME, respectively (no quantitative data were obtained for ATX-II because of the limited amount of standard available). Each of the values given above refers to the absorption maximum wavelength for each toxin (4 nm band width) and could be slightly enhanced by diode bunching[14].

Recoveries from rice samples spiked with different amounts of toxin (from 0.2 to 10 μg/g) ranged from 50 to 76%, as shown in Table I.

Fig. 2A shows the overall spectrochromatogram of a rice extract in which the spectra of alternariol and alternariol methyl ether are clearly identified. This furnishes an immediate example of the usefulness of three-dimensional plots in giving a first indication of the presence and identity of certain analytes possessing characteristic UV spectra. Obscured peaks were observed by rotating the viewing angle of the wavelength–time data plane and, eventually, zooming the zone of interest on the spectrochromatogram. Fig. 2B shows a typical example in which the examination of the sub-spectrochromatogram in the time window 11.6 – 13.0 min reveals the presence of ATX-II. Multi-signal chromatograms could be used to optimize, within a

TABLE I

RECOVERY OF SOME *ALTERNARIA* MYCOTOXINS FROM SPIKED RICE SAMPLES

Added (μg)	*Recovery (%) (mean ± S.D., n = 3)*			
	Altenuene	*Alternariol*	*Alternariol methyl ether*	*Altertoxin-I*
0.2	62.0 ± 6.7	72.0 ± 3.6	74.7 ± 3.8	–
1.0	51.6 ± 4.7	70.0 ± 3.2	76.0 ± 3.5	70.0[a]
10.0	51.0 ± 4.1	50.0 ± 7.1	73.5 ± 4.0	–

[a] Single determination.

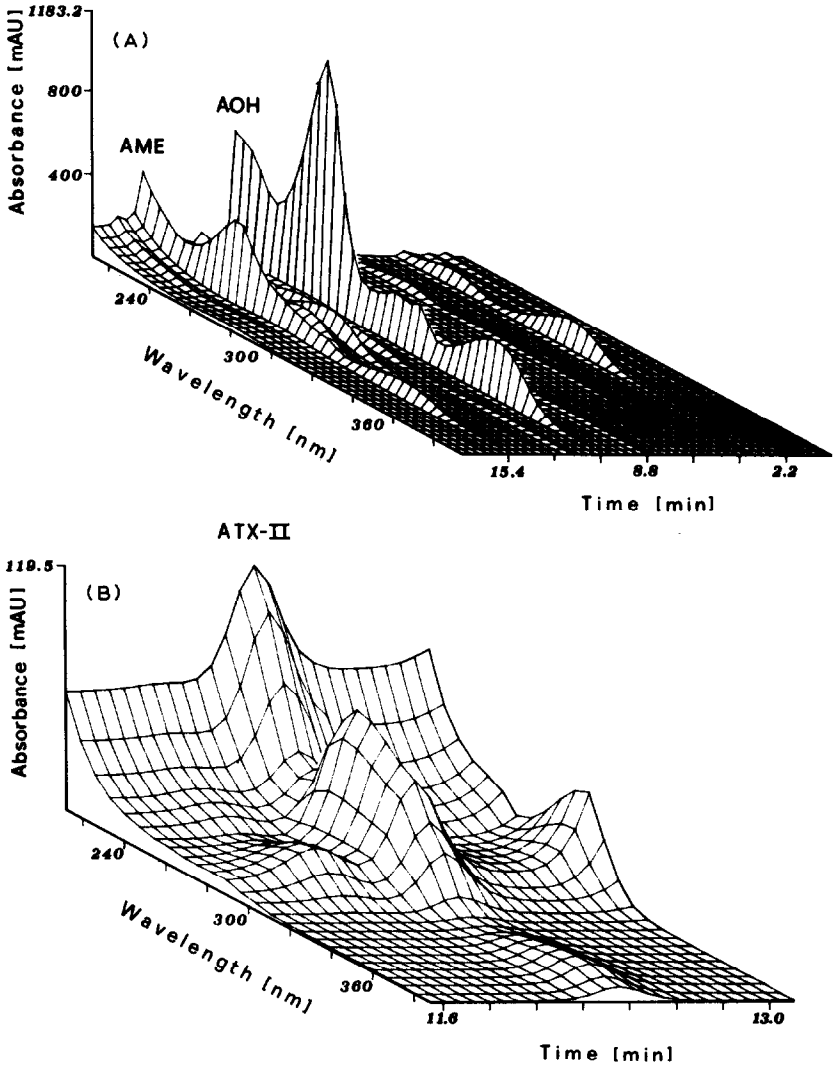

Fig. 2. (A) Overall spectrochromatogram for the extract of an artificially infected rice sample. (B) Sub-spectrochromatogram in the time region where altertoxin-II is eluted. Experimental conditions as described in the text except for spectral acquisition parameters; here 16 spectra/min were acquired.

single run, the sensitivity for the simultaneous detection of different toxins; this reduced considerably the analysis time compared with traditional methods[4,5,9] requiring multiple sequential injections at different wavelengths selected on the absorption maxima of each toxin.

A multi-signal chromatogram relevant to a rice sample artificially infected with *Alternaria* is shown in Fig. 3. Trace A (240 nm, 4 nm band width) may be regarded as a non-selective "total-peak chromatogram", and provides the maximum sensitivity for ALT; trace B at 257 nm (4 nm band width) optimizes the sensitivity for AOH, AME, ATX-I and ATX-II; trace C at 340 nm (40 nm band width) may be regarded as

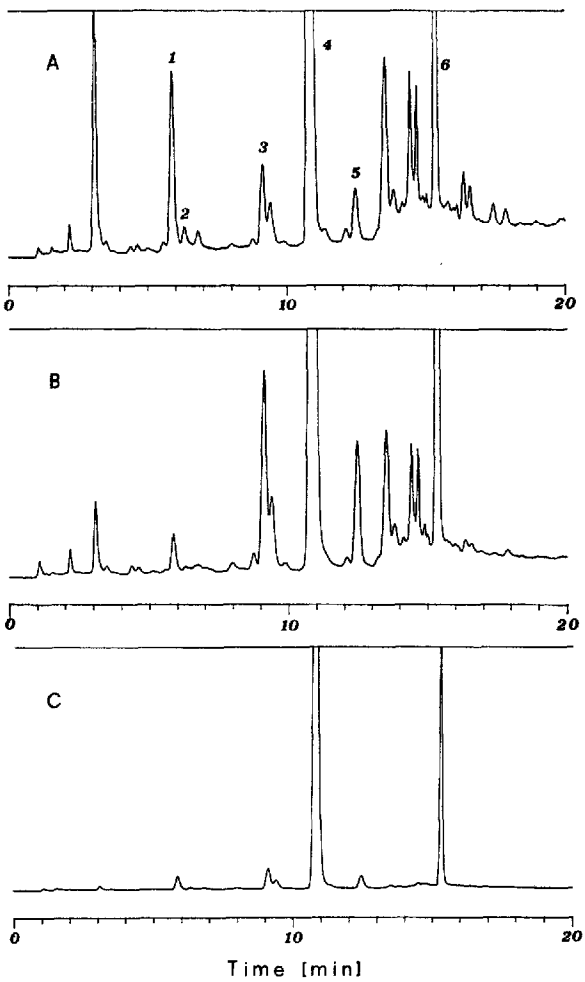

Fig. 3. Multi-signal plot of an extract of an artificially infected rice sample. Chromatographic conditions and spectral acquisition as specified under Experimental. Detection at 240 (trace A), 257 (trace B) and 340 nm (trace C). Spectral band width: 4 nm for traces A and B and 40 nm for trace C. Attenuation: 200 mAU full scale. Peaks: 1 = ALT (5 ppm); 2 = ALT isomer; 3 = ATX-I (3.7 ppm); 4 = AOH (200 ppm); 5 = ATX-II (2.3 ppm); 6 = AME (17.5 ppm).

a "toxin-selective" chromatogram because in this wavelength region the background contribution is minimized; it may be helpful for peak recognition with naturally contaminated samples.

For each peak a homogeneity test was performed by overlaying (after normalization) the spectra acquired on the apex and the ascending and descending parts of the peak. Provided no inhomogeneity was found, a peak identity test was performed by comparing the spectra of the unknown with the spectra of the standard (see Fig. 4 for an example).

Close inspection of spectrochromatograms of *Alternaria* culture extracts in the region around the elution times of altenuene revealed the presence of an additional

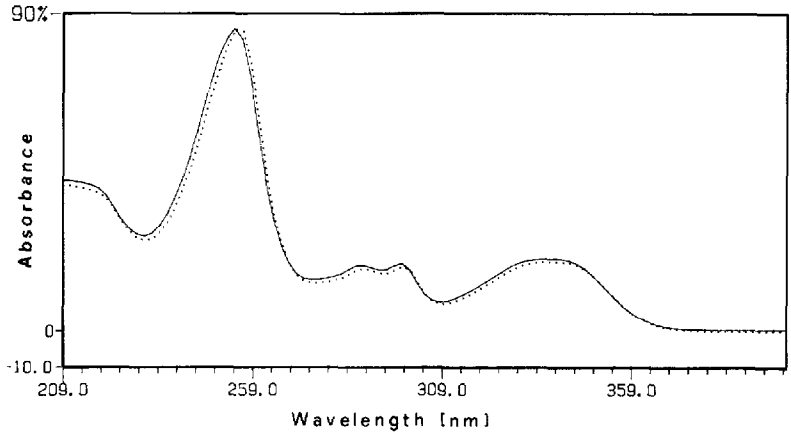

Fig. 4. Peak identity test for alternariol in a rice extract. Solid line, sample spectrum; dotted line, reference spectrum retrieved from a previously created archive.

compound with strictly similar UV spectral characteristics. We have isolated a suitable amount of the altenuene-like compound by preparative-scale HPLC from a culture of *Alternaria alternata* on rice, and identified it as an isomer of altenuene[15]. Note that a poor separation of the above compounds could have led to a failure of peak purity criteria, such as peak overlaying and ratio chromatograms, due to spectral similarity.

The method described here has been used successfully for profiling *Alternaria* mycotoxins in extracts of artificially infected rice, maize and tomato samples. For example, the chromatogram relevant to an extract of tomatoes containing 0.09 μg/g of ATX-I, 35 μg/g of AOH and 0.2 μg/g of AME is shown in Fig. 5. Fig. 6 shows the spectrochromatogram, acquired in the elution region of AOH, relevant to the extract of a naturally contaminated sunflower seed sample; in this sample AOH and AME were both unambiguously detected and quantitated (by the standard additions method) at levels of 0.36 and 0.13 μg/g, respectively. The above findings were corroborated by thin-layer chromatographic analysis of the same extract and demonstrate the applicability of the proposed method to real fungal-contaminated samples containing *Alternaria* metabolites at sub-ppm levels.

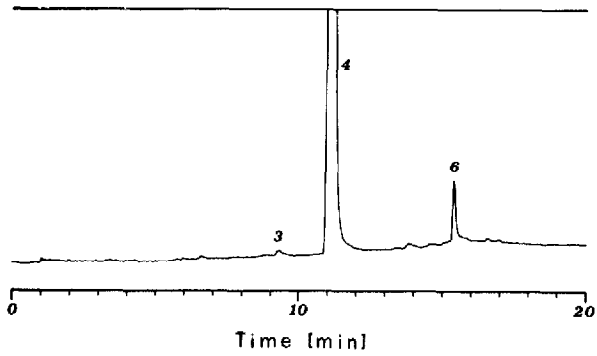

Fig. 5. Chromatogram relevant to the extract of an artificially infected tomato sample. Detection at 340 nm with a spectral band width of 40 nm. Attenuation, 10 mAU full scale. Peaks as in Fig. 3.

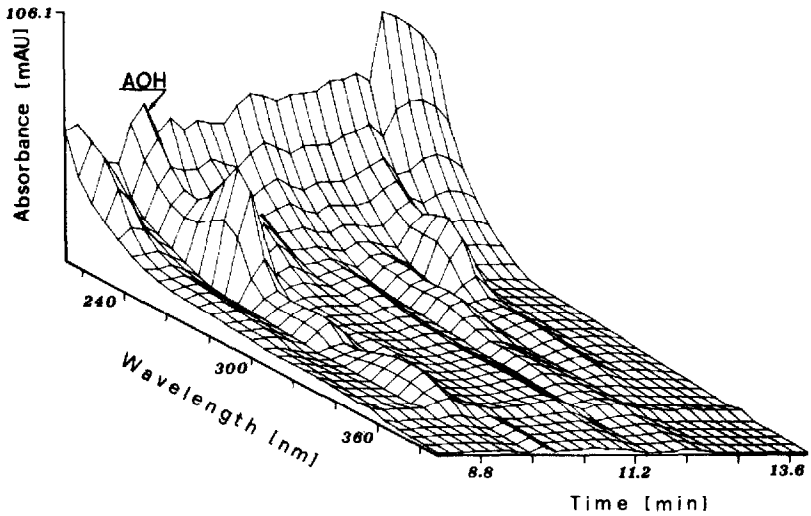

Fig. 6. Sub-spectrochromatogram (AOH elution region) relevant to the extract of a naturally contaminated sunflower seed sample.

CONCLUSIONS

The potential of diode-array detection for *Alternaria* mycotoxins profiling in food samples has been demonstrated. The multi-signal plotting capability may be an aid for a first identification (through the choice of selective wavelengths) and at the same time can optimize the detection sensitivity for compounds (or classes) having different absorption maxima. In addition, three-dimensional spectrochromatograms give UV spectra and the possibility of identification of those toxins having characteristic spectra. Spectra of unknown compounds can be compared with reference data previously stored and peak homogeneity can be checked.

Although UV spectra alone can rarely give an absolutely certain identification of a compound, a reasonable degree of confidence can be reached in most instances. Coupling of a diode-array UV detector to a functional group-specific detector, *e.g.*, electrochemical (in progress in our laboratory), should provide additional evidence (and sensitivity). This could avoid the need for sophisticated LC detection techniques such as mass spectrometry.

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