Journal of Chromatography, 540 (1991) 376–382
Elsevier Science Publishers B.V., Amsterdam

CHROM. 22 977

Short Communication

Selective determination of altertoxins by high-performance liquid chromatography with electrochemical detection with dual "in-series" electrodes

ANGELO VISCONTI* and ANGELA SIBILIA
Istituto Tossine e Micotossine da Parassiti Vegetali, Consiglio Nazionale delle Ricerche, Via G. Amendola 197/F, 70126 Bari (Italy)
and
FRANCESCO PALMISANO
Dipartimento di Chimica dell'Università, Campus Universitario 4, Trav. 200 Re David, 70126 Bari (Italy)
(First received August 2nd, 1990; revised manuscript received November 14th, 1990)

ABSTRACT

A selective method for the determination of altertoxin-I and altertoxin-II by high-performance liquid chromatography with electrochemical detection is described. Altertoxins were separated on a reversed-phase column with methanol–water containing 0.1 *M* sodium nitrate and 1 m*M* nitric acid (60:40) as eluent and detected with dual in-series electrodes operating in the "redox" mode (generator electrode +1.0 V, indicator electrode −0.1 V). The method was applied successfully to the determination of sub-ppm levels of altertoxins in samples of maize, rice and tomatoes infected by *Alternaria alternata*.

INTRODUCTION

Altertoxins are toxic metabolites produced by *Alternaria alternata*, a fungus which colonizes a wide variety of agricultural commodities in the field or during storage. These compounds are highly mutagenic to *Salmonella typhimurium* with or without metabolic activation [1]. Of a number of *Alternaria alternata* metabolites tested for phytotoxicity on tomato leaves and for antifungal activity *versus Geotrichum candidum*, altertoxin-I (ATX-I) and altertoxin-II (ATX-II) were found to be the most active [2]. They were lethal to mice at levels of 200 mg/kg body weight, and cytotoxic to HeLa cells [3]. The potent mutagenicity of altertoxins and the widespread occurrence of *Alternaria* isolates that may produce them pose a stringent need for selective and sensitive analytical methods suitable for screening for the occurrence of such mycotoxins in various foodstuffs. The techniques available for the determination of altertoxins are thin-layer chromatography and high-performance liquid chromato-

0021-9673/91/$03.50

graphy (HPLC) with UV or electrochemical detection [1,4–7]. A good sensitivity was achieved with amperometric detection [6], although the limit of detection in foodstuffs would be seriously affected because of the inadequate selectivity, which was similar to that achievable with UV diode-array detectors [5]. Selectivity was improved by using a dual coulometric detector operating in the "screen" mode [6].

In this paper we describe a new approach to the HPLC determination of altertoxins with electrochemical detection, which involves the use of dual in-series electrodes operating in the "redox" mode, and allows a better selectivity. Such an approach is made possible by the structural characteristics of these compounds (Fig. 1) having a biphenyl moiety liable to electrochemical oxidation and subsequent reduction at the dual in-series electrodes.

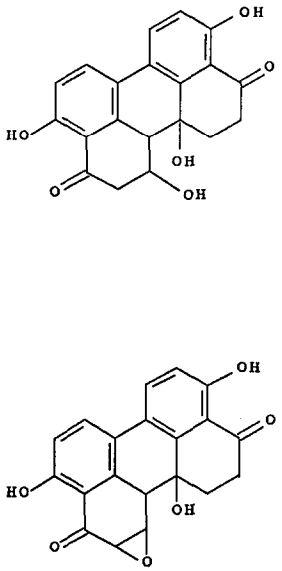

Fig. 1. Structures of altertoxin-I and altertoxin-II.

EXPERIMENTAL

Apparatus

An LKB (Bromma, Sweden) Model 2150 pump equipped with a Rheodyne Model 7125 injector and a LiChrospher 100 C_{18} (5 μm) reversed-phase column (125 × 4 mm I.D.) (E. Merck, Darmstadt, Germany) was used as the chromatographic system. A diaphragm-type pulse damper was placed between the pump and the injector in order to ensure a smooth, pulseless delivery of the mobile phase to the flow-sensitive electrochemical detector.

Coulometric detection was performed with an ESA (Bedford, MA, U.S.A.) Model 5100 Coulochem detector. An ESA Model 5011 dual-electrode analytical cell was used as the electrochemical transducer.

Reference standards

Toxin reference standards of ATX-I and ATX-II were prepared in our laboratory from a culture on rice [7] of *Alternaria alternata* strain ITM 334 (ATCC 66868) isolated from sunflower in Italy. The culture was extracted with methanol and (after filtration and addition of water) partitioned with methylene chloride. The methylene chloride extract was purified on a silica gel column (50 × 3 cm I.D.) eluted with methylene chloride–methanol (with increasing methanol concentration). Fractions containing altertoxins were submitted to further purification by HPLC on a semi-preparative LiChrosorb RP-18 (7 μm) column (250 × 25 mm I.D.) eluted with methanol–water (90:10 for ATX-II; 80:20 for ATX-I). Pure toxins (>98%, checked by HPLC) were obtained after evaporation of methanol and freeze-drying. The identities of the purified toxins were confirmed by comparison with authentic standards supplied by M. E. Stack (Food and Drug Administration, Washington, DC, U.S.A.). Stock solutions were prepared in methanol and stored in the dark at 4°C. Diluted standards were prepared just before use by addition of mobile phase.

Extraction of samples

Cultures of *Alternaria* on maize, rice and tomatoes were prepared and extracted as described previously [5,7]. Briefly, 50 g were homogenized in a blender for 2 min with 75 ml of methanol. The homogenate was filtered and 40 ml of filtrate were clarified with 80 ml of 20% aqueous ammonium sulphate and filtered again. A volume of filtrate equivalent to 20 g of sample (90 ml) was extracted twice with 5 ml of methylene chloride and the combined extracts were evaporated to dryness. The residue was reconstituted to 1 ml with methanol and analysed by HPLC.

HPLC

The mobile phase used at a flow-rate of 1 ml/min for the determination of altertoxins in maize, rice and tomatoes was methanol–water containing 0.1 *M* sodium nitrate and 1 m*M* nitric acid (60:40, v/v). Mobile phase was used to wash the column for 20 min after each HPLC run (10 min). The injection volume was 5 μl. All solvents were of HPLC grade. The mobile phase was filtered through a 0.45-μm membrane and vacuum degassed before use.

The ESA Model 5011 analytical cell potential was as follows: upstream (generator) electrode, +1.00 V; downstream (indicator) electrode, −0.10 V. All potentials are referred to the ESA proprietary reference electrode.

RESULTS AND DISCUSSION

The selectivity of conventional anodic electrochemical detection in HPLC often tends to be over-emphasized. It should be pointed out that the selectivity deteriorates rapidly as the applied potential moves (as required for *Alternaria* metabolites) towards the anodic limit possible under the actual operating conditions. Dual in-series electrode detection in the "redox" mode may overcome the problem in some favourable cases [8]. In this technique, analytes are electrochemically oxidized by the upstream electrode (generator) and the products reduced at the downstream electrode (collector). The result is an improved selectivity for those compounds having collection efficiencies (downstream/upstream peak current ratio) that are significantly higher

than those of coeluting compounds. The collection efficiency depends on the stability of the oxidation product, the cell geometry and the actual experimental conditions. An essential requirement for applying such a technique is that the generated species should have a lifetime which is long relative to the time necessary to travel from the upstream to the downstream detector. Many reactions that behave irreversibly when examined by other electroanalytical techniques (cyclic voltammetry, etc.) display a high degree of reversibility in the redox mode because the transit time is relatively short [8]. The transit time in our apparatus was a few milliseconds, short enough to allow a good collection efficiency for the determination of altertoxins.

Fig. 2 illustrates the hydrodynamic voltammograms obtained for ATX-I, ATX-II, alternariol (AOH) and alternariol methyl ether (AME). In order to establish the optimum detection potentials for our purposes, voltammograms were generated on the downstream electrode, while the potential of the upstream electrode was held at 1.0 V [6]. Fig. 2 reveals that ATX-I and ATX-II are the only toxins showing a high collection efficiency (0.60 and 0.55, respectively), while AOH and AME have a value of 0.05. The maximum collection efficiency expected (0.8) for the cell used in this work was derived using hydroquinone as a test compound.

The 10-fold higher collection efficiency for altertoxins compared with alternariols shows the high selectivity of this technique for altertoxins compared with other metabolites of the same fungal species (*A. alternata*) which are normally produced in cultures (and are expected to co-occur in contaminated samples) at much higher levels.

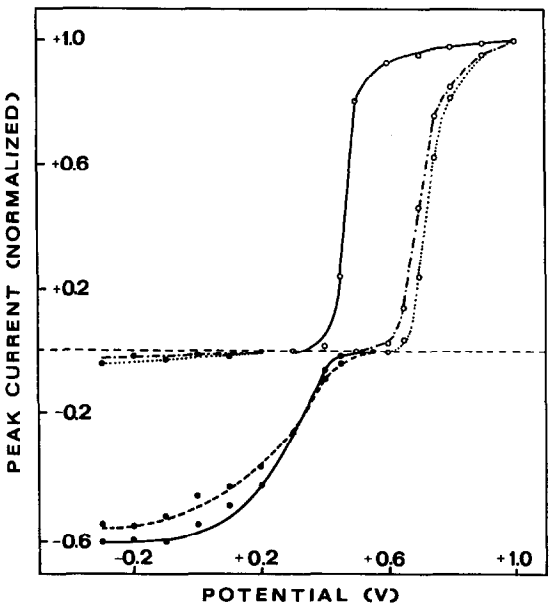

Fig. 2. Hydrodynamic voltammograms of alternariol (– · –), alternariol methyl ether (· · · ·), altertoxin-I (———) and altertoxin-II (– – –) generated with a dual-electrode coulometric detector. (○) Anodic wave at the upstream electrode E1 (anodic waves for ATX-I and ATX-II overlap); (●) cathodic wave at the downstream electrode E2 with electrode E1 maintained at +1.00 V. All current responses are normalized to the value at +1.00 V. Injected amounts: 50 ng. Potential values are referred to the ESA proprietary reference electrode. Other conditions as specified in the text.

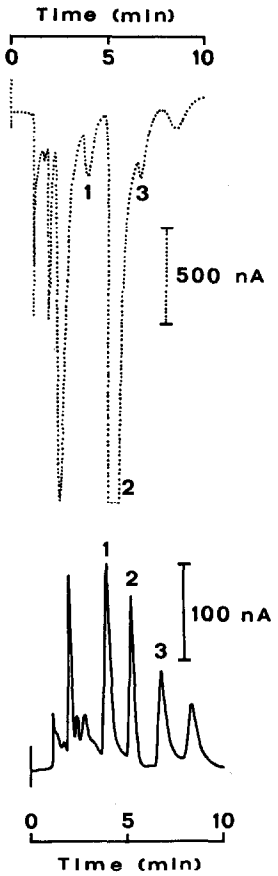

Fig. 3. Chromatograms for 5 μl of a 100-fold diluted rice extract containing 14 ppm of ATX-I and 10 ppm of ATX-II. Upper and lower chromatograms refer to the upstream and downstream electrode, respectively. Peaks: 1 = ATX-I; 2 = AOH; 3 = ATX-II. Chromatographic conditions as specified in the text.

A typical example of such a situation is shown in Fig. 3, in which chromatograms relevant to a rice culture extract obtained at the upstream and downstream electrodes are represented. Isocratic elution with single-electrode anodic detection at +1.00 V (comparable to the upper track in Fig. 3) was unsatisfactory for the analysis of this sample, which contained large amounts of alternariol, causing ATX-II to elute as a poorly resolved shoulder.

The optimum working potentials were established as +1.00 V and −0.1 V for the upstream and downstream electrodes, respectively. Using these conditions, linearity of the detector current *vs.* concentration plot was observed for about three decades (0.24–500 ng for ATX-I and 0.42–530 ng for ATX-II), and on-column detection limits at a signal-to-noise ratio of 3 were 30 pg and 100 pg for ATX-I and ATX-II, respectively. Signals (peak heights) relevant to replicate injections of reference standards during four consecutive days (three injections per day) at two

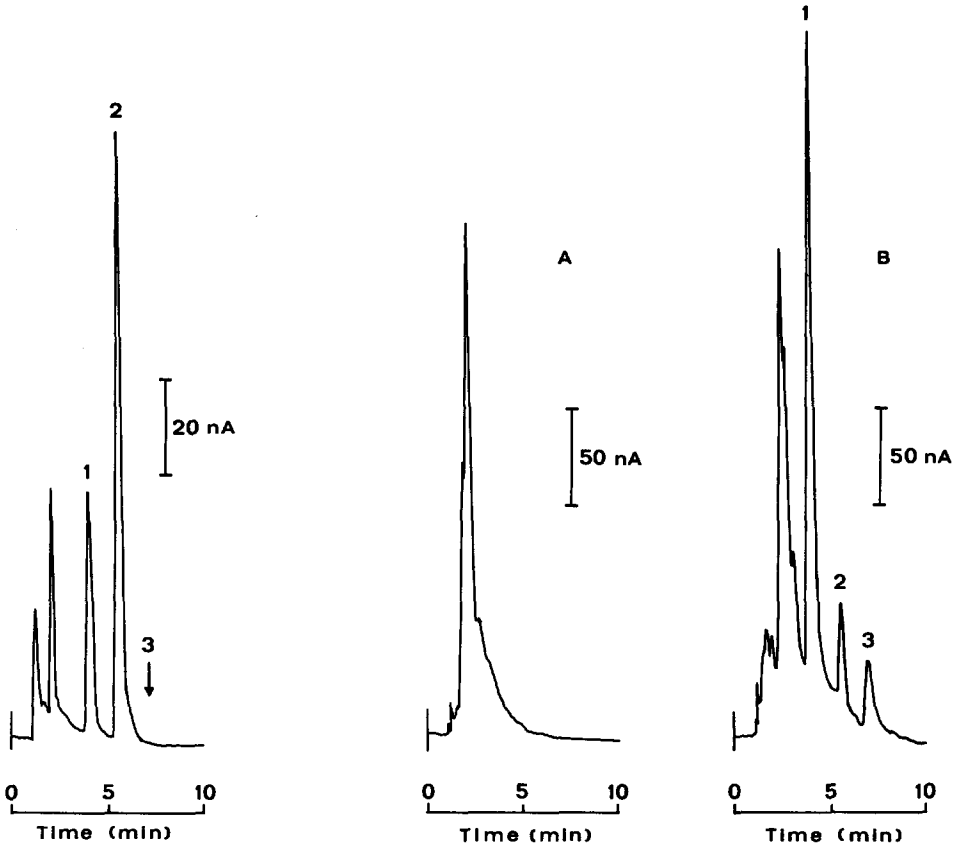

Fig. 4. Chromatogram for 5 μl of a tomato extract containing 35 ppb of ATX-I. Peak identification and chromatographic conditions as in Fig. 3.

Fig. 5. Chromatograms for 5 μl of a 10-fold diluted extract of maize: (A) blank and (B) containing 2 ppm of ATX-I and 270 ppb of ATX-II. Peak identification and chromatographic conditions as in Fig. 3.

different concentrations (5 and 50 ng) showed relative standard deviations of 4.8% and 5.5% for ATX-I and 3.3% and 3.9% for ATX-II, respectively.

This method has been successfully used for monitoring the presence of ATX-I and ATX-II at sub-ppm levels in extracts of artificially infected maize, rice and tomato samples. For example, the chromatogram relevant to a tomato extract containing 35 ppb of ATX-I is shown in Fig. 4. Fig. 5A and B show chromatograms relevant to an extract of blank maize and maize containing 270 ppb of ATX-II and 2 ppm ATX-I, respectively. Notwithstanding the complexity of the sample matrices, very clean chromatograms showing the absence of coeluting interferents are obtained, demonstrating the selectivity of the method for the determination of altertoxins.

The method could prove particularly useful for the determination of ATX-I and ATX-II in various foodstuffs owing to its selectivity, rapidity, detection capabilities and reduced instrumental requirements (*e.g.*, isocratic elution).

REFERENCES

1 M. E. Stack and M. J. Prival, *Appl. Environ. Microbiol.*, 52 (1986) 718.
2 A. Visconti, A. Bottalico and M. Solfrizzo, in A. Graniti, R. D. Durbin and A. Ballio (Editors), *Phytotoxins and Plant Pathogenesis*, Springer, Berlin, 1989, p. 457.
3 R. W. Pero, H. Posner, M. Blois, D. Harvan and J. W. Spalding, *Environ. Health Perspect.*, (1973) 87.
4 J. E. Schade and A. D. King, *J. Food Prot.*, 47 (1984) 978, and references cited therein.
5 F. Palmisano, P. G. Zambonin, A. Visconti and A. Bottalico, *J. Chromatogr.*, 465 (1989) 305.
6 F. Palmisano, P. G. Zambonin, A. Visconti and A. Bottalico, *Chromatographia*, 27 (1989) 425.
7 A. Visconti, A. Logrieco and A. Bottalico, *Food Addit. Contam.*, 3 (1986) 323.
8 *Model 5100 Instruction Manual*, ESA, Bedford, MA, 1985.