Determination of *Alternaria* Mycotoxins in Foodstuffs by Gradient Elution Liquid Chromatography with Electrochemical Detection

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Key Words

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Summary

The electroactivity of major *Alternaria* mycotoxins has been explored in order to devise a liquid chromatographic-electrochemical detection (LC-EC) method for the determination of these toxic metabolites in fungal cultures and in naturally contaminated foodstuffs. Alternariol, alternariol methyl ether, altertoxin-I and altertoxin-II have been found to be electroactive and could be detected at sub-nanogram levels. Advantages and limitations of dual electrode (screen mode) coulometric detection and single electrode amperometric detection are presented and discussed. The feasibility of electrochemical detection in gradient elution liquid chromatography as well as its applicability to the analysis of contaminated foodstuffs is demonstrated.

Introduction

The genus *Alternaria* is widely distributed in soil and decaying organic materials and is often parasitic on plants. Moulds of this genus require a relatively high moisture content for growth and cause spoilage of commercially important foodstuffs such as fruit and vegetables, both in the field and after harvest.

Alternaria produce several metabolites possessing, some degree of toxicity to mammalian and bacterial cells as well as to higher plants and laboratory animals [1–3]. Although *Alternaria* mycotoxins generally occur only in trace amounts in unspoiled foods, they could equally pose a threat to human health due to their chronic toxicity. The most common procedure for quantitative analysis of *Alternaria* metabolites is thin layer chromatography [4]. Gas-liquid chromatography (GLC) has also been used to separate and detect altenuene (ALT), alternariol (AOH), alternariol methyl ether (AME) and tenuazonic acid usually as the corresponding trimethylsilyl derivatives [4]. Flame ionization detectors give, however, only poor detection limits, often unsuitable for naturally contaminated samples. The use of selective and sensitive detectors, other than mass spectrometry [5, 6], has not yet been explored.

High performance liquid chromatography (HPLC) possesses a highly desirable advantage over GC in that no derivatization step is required. Most HPLC applications have been performed on octadecylsilane columns with conventional UV detection [7–11]. The best detection limits reported are in the low ng (on column) range. Fluorescence detection is also possible for naturally fluorescent metabolites but, surprisingly, only sporadic applications have been described [9, 12].

In the authors' laboratory the potential of multichannel diode array UV detection has been recently explored [13]. AOH, AME, ALT, isoaltenuene (iso-ALT, a new metabolite recently isolated [14]), altertoxin-I (ATX-I) and altertoxin-II (ATX-II) have been (simultaneously) profiled in a variety of matrices, by gradient elution reversed phase liquid chromatography.

Liquid chromatography – electrochemical detection (LC-EC), which has gained a widespread acceptance [15] because of its sensitivity and selectivity, is still poorly employed in mycotoxins research and, as far as we know, has not been used in the field of *Alternaria* toxins. This is quite surprising because most *Alternaria* metabolites of major concern to human health (e.g. the dibenzo-(α)-pyrone and perylenquinone derivatives shown in Figure 1) possess phenolic moieties and should be, in theory, electrochemically active.

In this paper the first LC-EC approach to *Alternaria* mycotoxins determination in foodstuffs is reported. AOH, AME, ATX-I and ATX-II have been found anodically electroactive and can be detected at level of 10–50pg on column (isocratic elution) in both coulometric and amperometric mode (the term "coulometric" is used to indicate an amperometric detector with a porous graphite electrode allowing 100% conversion efficiency). The feasibility of gradient elution

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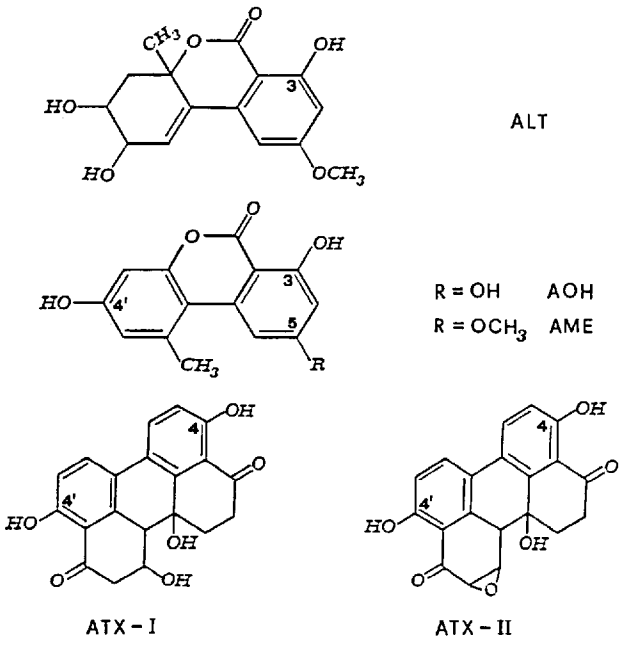

Fig. 1

Chemical structures of the *Alternaria* metabolites studied in the present work. Abbreviations used: ALT = altenuene, AOH: alternariol; AME: alternariol methyl ether; ATX: altertoxin.

LC-EC for profiling electroactive toxins in very complex matrices such as fungal cultures and naturally contaminated foodstuffs is also demonstrated.

Experimental

Apparatus

A Perkin Elmer (Norwalk, CT, USA) mod. 3B dual pump module equipped with a Rheodyne 7125 injector and a reversed phase column (Spherisorb ODS-II 125 x 4.6mm – 5μm packing – Bishoff, Leonberg, FRG) was used as the chromatographic system. Isocratic work was carried out on a system composed of an LKB 2150 pump (LKB-Produkter AG, Bromma, Sweden), a Rheodyne 7125 injector and a reversed phase column (LiChrospher 100 C18 125 x 4mm – 5μm packing – E. Merck, Darmstadt, FRG). In both cases a diaphragm type pulse dampener was placed between pump(s) and the injector in order to ensure a smooth, pulseless delivery of the mobile phase to the flow sensitive electrochemical detector.

Amperometric detection was accomplished by using a BAS (Bioanalytical System, West Lafayette, IN, USA) mod. TL5A thin-layer cell (3mm glassy carbon working electrode) controlled by a PAR 174A polarographic analyser (EG&G Princeton, NJ, USA). Coulometric detection was performed with an ESA mod. 5100 Coulochem detector (ESA Inc., Bedford, MA, USA). A dual electrode ESA mod. 5011 Analytical cell was used as the electrochemical transducer. An ESA mod. 5020 guard cell placed before the injector was used to pre-electrolyse the mobile phase. The guard cell was also used for amperometric detection.

Chemicals

Toxin reference standards were obtained as described elsewhere [11]. Stock solutions of selected toxins were prepared in methanol and stored in the dark at 4°C. Diluted standards were prepared just before use by addition of mobile phase.

All solvents used were HPLC grade. The other chemicals were analytical reagent grade. The mobile phase was filtered through a 0.45μm membrane and vacuum degassed before use.

Toxins Production and Extraction

Cultures of *Alternaria* on maize and rice substrates were prepared as previously described [11]. Naturally contaminated mandarin samples and sunflower seeds were collected in the field in southern Italy, and extracted according to a procedure described elsewhere [13].

Chromatographic Conditions

The following elution programs and solvent systems were used.

Coulometric detection: 60% solvent A for 5min, to 85% in 10min then isocratically for 5min. Solvent A: methanol; solvent B: 0.05M phosphate buffer (pH 6.0).

Amperometric detection: from 50% to 55% solvent A in 10min, to 80% in 25min then isocratically for 5min. Solvent A: methanol – 0.025M phosphate buffer pH 5.8 (80 + 20) v/v; solvent B: methanol – 0.025M phosphate buffer pH 6.0 (20 + 80) v/v.

Amperometric cell potential: 1.2V vs. Ag/AgCl. Coulometric cell potential: 0.5 and 1.0V (vs. ESA proprietary reference electrode), for downstream and upstream electrodes, respectively.

Guard cell potential: 1.05V vs. ESA reference electrode.

Flow rate was 1.0mL/min. Temperature: ambient.

Results and Discussion

Hydrodynamic Voltammetry of *Alternaria* Toxins

In order to establish the electroactivity of AOH, AME, ATX-I, ATX-II and ALT and the optimum detection potential for LC-EC purposes, hydrodynamic voltammograms were generated on both the coulometric and amperometric detectors by sequential injections of authentic standards, under isocratic conditions (60% methanol – 40% 0.05M phosphate buffer pH 6.0). The most important features of such voltammograms are the $E_{1/2}$ value e.g. the potential value at half the limiting current (which relates to a thermodynamic parameter – the standard potential of the redox couple) and the shape of the current voltage curve (which is influenced by electron transfer kinetics).

Hydrodynamic voltammograms revealed that AOH, AME, ATX-I and ATX-II are electro-oxidized in a limited potential window from 0.5 to 1.0V (Figure 2). Altenuene was the most difficult compound to oxidise since the foot

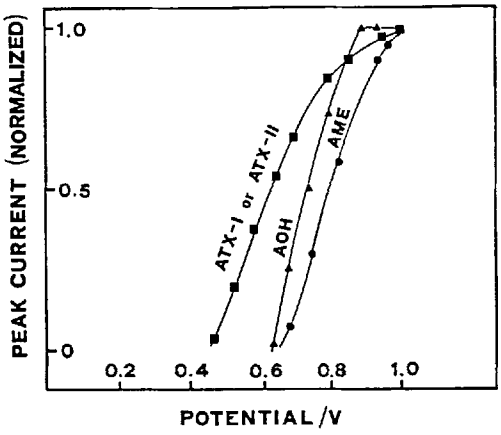

Fig. 2

Hydrodynamic voltammograms of AOH, AME and ATX-I (or ATX-II) generated with a coulometric detector. Injected quantities: 5ng. Eluent: 0.05M phosphate buffer pH 6.0/methanol (60:40) v/v. Flow rate: 1mL/min. Current responses are normalized to the value at +1.0V. Potential values are referred to the ESA proprietary reference electrode.

of the voltammogram just started to develop at a potential around +1.2V, i.e. near the anodic limit of the electrode material (electrochemistry of altenuene appears, therefore, to be of marginal interest for analytical purposes).

A detailed study on the electro-oxidation mechanism of these compounds would be of interest but is beyond the aim of the present work and is also hampered by the limited number of certified standards generally available. However the following general conclusions can be drawn. $E_{1/2}$ values, shifted towards anodic potentials by decreasing the pH, indicated a pH dependent process. This is in agreement with the general finding that in acidic media, non-ionized phenols usually undergo an oxidation step (with the abstraction of two electrons and one proton) to give a phenoxonium ion which may undergo further chemical reactions e.g. dimer (or polymer) formation and/or nucleophilic attack from solvent [16–18]. On the other hand this suggests that the pH value of the mobile phase must be a compromise between electrochemical requirements, stationary phase stability and control of secondary (ionization) equilibria. The optimized pH value found in this study is around 6.

The poor electroactivity of ALT can be tentatively explained on the basis of a poor stabilization of the phenoxonium intermediate. In fact in the ALT molecule (see Figure 1) there is no meta or para substituent which can stabilize the intermediate formed by the oxidation of the phenolic group at C(3) which is the only electroactive moiety. The opening of a six membered ring deriving from hydrogen bonding between C(3)–OH and the lactone C=O group may even represent an additional factor hindering the oxidation step. Similar arguments could explain the AME electroactivity which may be ascribed to the –OH group at C(4′), in the para position in the aromatic ring which helps to stabilize the intermediate. The similarity in the $E_{1/2}$ values for AME and AOH again lead to the conclusion that the –OH group at the C(4′) is responsible for the electroactivity of AOH (the –OH group at C(5) is less favoured and its oxidation should eventually occur in a second step at a potential more anodic than those explored). The lowest $E_{1/2}$ value observed for altertoxins could again be explained by the presence of a 4-4′ biphenyl moiety (see Figure 1) whose oxidation product can be stabilized by quinoid structures [19].

LC-Coulometric Detection

Once the electroactivity of the various compounds had been established, the optimum working potential and linearity of the detector response was ascertained. For example, a calibration plot for AOH, in the range 0.1–50ng on-column (isocratic elution), revealed a linear response (correlation coefficient better than 0.995) with an intercept not significantly different from zero (at 95% confidence level) and a typical sensitivity of 40nA/ng. The detection limit was estimated to be around 50pg on-column, i.e. about two orders of magnitude better than UV detection. This improvement could be also beneficial, in complex matrices such as fungal contaminated foodstuffs where detection limits are often dictated by chemical noise rather than instrumental sensitivity.

Isocratic elution, however, proved unsatisfactory for the analysis of real samples. A suitable gradient elution program had to be developed bearing in mind the limited compatibility of electrochemical detection with gradient elution [20, 21]. For a given applied potential and mobile phase composition, the major factors influencing background current (and base line shift) in electrochemical detection are mobile phase purity and ionic strength or pH variation occurring during gradient elution [22]. The use of a pre-electrolysis (guard) cell helped to remove electroactive impurities in the mobile phase thus reducing background current. The particular design of the ESA coulometric cell, in which there is no separation between test and reference electrodes [23] reduced considerably the uncompensated resistance and the sensitivity to ionic strength (and conductivity) changes generated by the gradient analysis [21]. pH changes remained the main factor influencing the base line shift. Figure 3 shows a chromatogram of a mixture of ATX-I, AOH, ATX-II and AME standards in which the base line drift is quite acceptable at least at the specified instrumental sensitivity setting which is more than adequate for the analysis of fungal cultures and/or heavily contaminated samples (vide infra).

Enhanced selectivity was obtained by dual electrode coulometric detection in "screen" mode [23], with the upstream (coulometrically efficient) and downstream (indicator) electrodes held at +0.5V and +1.0V respectively. In this way only the mycotoxins of interest and those interfering compounds electroactive in the fixed potential window were detected, while the more easily oxidizable compounds were quantitatively removed by the upstream electrode. Figure 4 shows a typical gradient elution chromatogram of an extract of *Alternaria* cultures on rice obtained in screen mode detection.

A major drawback of coulometric detection (isocratic or gradient elution) was a pronounced tailing of the chro-

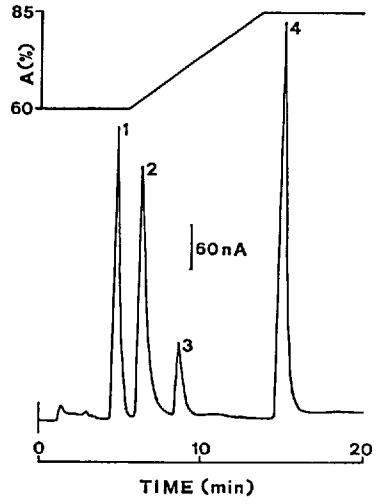

Fig. 3

Chromatogram of a mixture of toxin standards. Peak identification: **1** = ATX-I (10ng); **2** = AOH (10ng); **3** = ATX-II (4ng) and **4** = AME (30ng). The solvent program is also shown in the upper graphic. Solvent A: methanol; solvent B: 0.05M phosphate buffer (pH 6.0). Dual coulometric electrode detection: upstream electrode + 0.5V, downstream electrode + 1.0V. Guard cell maintained at + 1.05V. Potential values are referred to the ESA proprietary reference electrode.

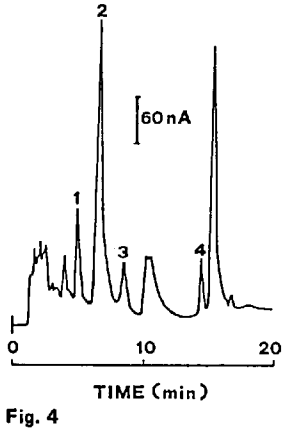

Fig. 4

Chromatogram of a rice culture extract containing 0.1ppm ATX-I, 1.1ppm AOH, 0.43ppm ATX-II and 0.65ppm AME. (2μL of a tenfold diluted extract were injected). Peak identification and chromatographic conditions as specified in Figure 3.

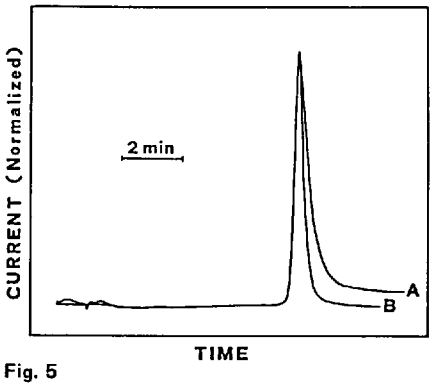

Fig. 5

Chromatograms for AOH under identical experimental conditions except for the detector type. Curve A: coulometric; curve B: amperometric. Chromatographic conditions as in Figure 2.

matographic peaks for the toxins of interest. The possibility that peak tailing was of chromatographic origin was excluded since it was not observed when the coulometric detector was replaced by a UV or amperometric detector (see Figure 5). Significant extra-column peak broadening effects (due to coulometric cell and connecting tubing) were also excluded since the degree of tailing was different for each toxin peak, being particularly pronounced for AOH. The AOH peak obtained with a coulometric detector, showed an asymmetry factor (measured at 10% peak height) 2.5 times greater than the one obtained with an amperometric cell under identical experimental conditions. We have no definite evidence about the origin of this detector artifact but tentatively ascribe it to surface phenomena, related to the electro-oxidation process and to the nature of the electrode material and cell geometry. It is well known [16] that electrochemical oxidation of phenolic compounds is complicated by strong adsorption phenomena on a variety of electrode materials. Evidence can even be inferred from the base line shift observed after AOH elution in spite of the isocratic conditions (see Figure 5). This is clearly a memory effect symptomatic of a modification of the electrode surface which can be restored later, due to washing by the mobile phase. The less pronounced peak tailing observed with the amperometric cell could be due to the nature of the electrode material (glassy carbon instead of porous graphite), as well as to the different electrode area and different flow pattern (e.g. enhanced washing characteristics of the cell).

LC-Amperometric Detection

The same approach used for coulometric detection was initially adopted, that is construction of hydrodynamic voltammograms, verification of the detector linearity and investigation of detection limits. The optimum detection potential was + 1.2V vs. Ag/AgCl and linearity was observed over about three decades (typically in the range 0.1 to 100ng on-column). Figure 6 shows some chromatograms for ATX-I near to the detection limit illustrating the sensitivity under isocratic elution conditions.

For the analysis of food samples the gradient program previously used for coulometric detection needed to be reoptimized in order to deal with interference problems which, in this case, are more pronounced due to the absence of a screen electrode. Moreover, due to its design, the amperometric cell had more pronounced IR drop problems requiring more efforts in optimizing the solvents composition in order to reduce changes in conductivity (and pH) of the mobile phase during gradient elution. During the solvent program used (see "Chromatographic Conditions") the maximum changes in the apparent pH and specific conductance where 0.5 pH units and − 120μmho/cm, respectively. The potential of LC-amperometric detection technique can be inferred from Figure 7 which shows the chromatogram from the extract of naturally contaminated sunflower seeds containing AOH and AME at level of 0.35 and 0.13μg/g respectively (concentrations were determined by standard addition). It is worth noting the reduced shift in the base line (contained within a few nAmps) and the

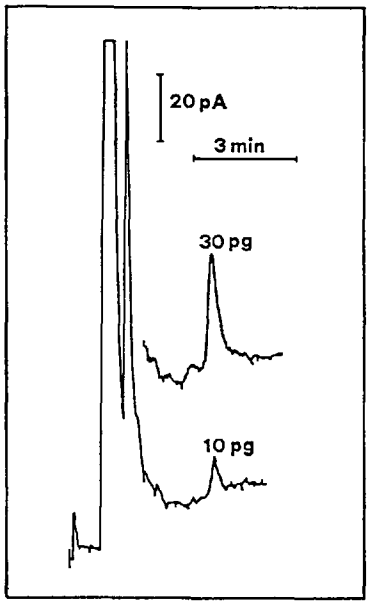

Fig. 6

Examples of chromatograms near to the detection limit for ATX-I. Glassy carbon amperometric detector operated at +1.2V vs. Ag/AgCl. Isocratic elution with a mobile phase composition as specified in Figure 2.

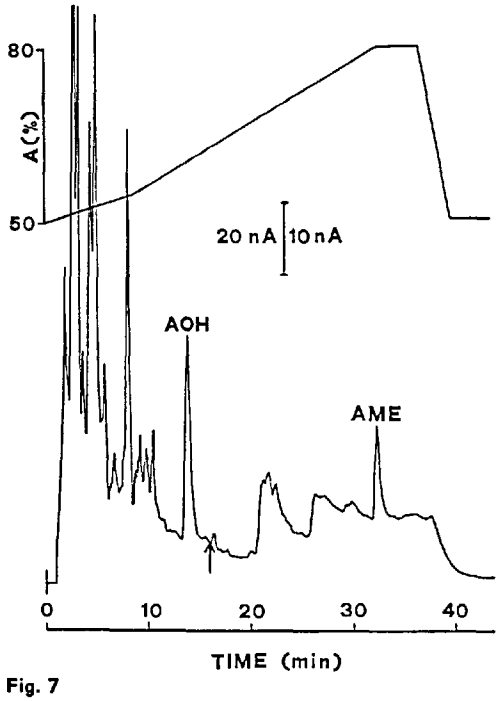

Fig. 7

Gradient elution-amperometric detection of AOH (0.35ppm), and AME (0.13ppm) in the extract of naturally contaminated sunflower seeds. Column: Spherisorb ODS-II (125 X 4.6mm), 5μm packing. Flow rate: 1mL/min. Other conditions as specified in "Experimental". The solvent program used is shown in the upper part of the figure. The arrow indicates a change in the detector sensitivity. The current mark is 20nA on the left side of the arrow and 10nA on the right.

return to its initial value (e.g. no poisoning or memory effect of the electrode surface) after the gradient run. The method has been also applied to the detection and quantitation of AOH and AME at sub ppm level in the extract of a naturally contaminated mandarin sample.

Conclusions

The potential of gradient LC-EC for the determination of *Alternaria* metabolites in complex matrices such as fungal cultures and/or naturally contaminated foodstuffs has been demonstrated. Dual electrode coulometric detection (screen mode) offers, apart from sensitivity, a high degree of selectivity but suffers from severe peak tailing produced by the detector. Single electrode amperometric detection, although not as selective as coulometric detection, appears equally promising. Its potential could be enhanced (work in progress) by different approaches such as: 1) the use of a coulo-amperometric cell (e.g. a coulometrically efficient, porous graphite, screen electrode coupled to a glassy carbon indicator electrode) and 2) the use of a single electrode LC-EC coupled to a column switching technique. Both approaches should enhance selectivity; the second one could even permit the use of isocratic elution which, in turn, should fully benefit from the very low detection limits offered by electrochemical detection. This could prove particularly useful for ultra-trace determination of the altertoxins which have been shown to be powerful mutagens.

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