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Stability and determination of aflatoxins by high-performance liquid chromatography with amperometric detection

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Abstract

A method based on reversed-phase high-performance liquid chromatography (RP-HPLC) with amperometric detection with a glassy carbon electrode at a constant potential of 1.4 V is reported for the separation and identification of aflatoxins B_1, B_2, G_1 and G_2 in a model mixture. The chromatography was performed on a PAH-Baker column with a ternary mobile phase containing methanol, acetonitrile and aqueous $LiClO_4$ electrolyte. Aflatoxin G_1 showed the highest electroactivity in the compound series studied. Calibration curves of aflatoxins G_1 and B_2 were linear up to 0.2 and 0.3 mmol/l, respectively. Sensitivity varied between 7 and 10 ng for the different aflatoxins. The combination of different HPLC detectors in the analysis of these compounds was applied to investigate the stability of aflatoxins G_1 and B_2.

Keywords: Aflatoxins; Electrochemical detection

1. Introduction

The discovery of serious aflatoxicosis in farm animals and the presence of aflatoxins in many food products triggered a worldwide effort to develop analytical methods for these compounds. Many procedures have been devised for extracting and isolating commonly occurring mycotoxins from feed and food products. Simple, rapid, sensitive and specific chromatographic methods [1,2] are now replacing complicated and less effective spectrometric methods for determining aflatoxins. Both normal-phase (NP) and reversed-phase (RP) high-performance liquid chromatography (HPLC) have been used in conjunction with UV absorption, fluorescence, mass spectrometry (MS) and amperometric detection. In combination with fluorescence detection, HPLC is, under optimum conditions, about 30–40 times more sensitive than UV detection for aflatoxins. Sensitivity may be enhanced to the nanogram level by converting the aflatoxins to more intensely fluorescent derivatives by pre-column derivatization with trifluoroacetic acid [3,4] or post-column derivatization with iodine or bromine [5,6]. These methods have become standard techniques, reviewed in [7], with a performance in the case of iodine derivatization, for example, of 0.5 ng/ml for aflatoxins B_2 and G_2 with 250 μl injection volumes [8], 7 pg/ml in urine [9], 3 ng/g of dust [10], or giving reproducible results for all four aflatoxins at concentrations over a range from 3 to 11 ng/g of corn [11]. By comparing pre- and post-column derivatiza-

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tion methods, a detection limit almost three times greater (0.3 μg/kg for aflatoxin B_2) was reached by prederivatization [12].

Chromatographic conditions play an important role in fluorescence intensity. To this, one can add that it also depends on temperature and reagent flow in the derivatization procedures. In order to enhance fluorescence detection while avoiding chemical derivatization and reducing the time required, post-column photo-derivatization has also been tested [13]. Here, detection limits were 1 and 0.25 μg/kg for aflatoxins B_1 and B_2, respectively, using 20 μl injections.

Electrochemical detection has become popular for trace analysis due to its high sensitivity and excellent selectivity. Pulsed amperometric detection, using a mercury electrode, made it possible to detect 5 ng of all four aflatoxins when 20 μl were injected on the column [14]. Based on a polarographic study of these compounds [15], aflatoxin B_1 was determined in food with a detection limit of 2.5 μg/kg for 200 μl injection volumes by using reductive voltammetric detection in the differential pulse mode with a static mercury electrode at −1.25 V vs. Ag/AgCl [16]. With a porous graphite electrode, both amperometric and coulometric mode detection has been employed in the determination of *Alternaria* mycotoxins at picogram levels [17] and, as far as we know, it has not been used in the field of *Aspergillus flavus* mycotoxins, i.e. aflatoxins.

2. Experimental

2.1. Equipment and materials

HPLC separations were performed on a Beckman System Gold HPLC apparatus with a Model 126 pump, a Model 507 autosampler and the following detectors connected in series: a Model 166 variable wavelength detector, a Shimadzu Model RF-551 spectrofluorimetric detector and a Model PAD-2 Dionex pulsed amperometric detector. The amperometric detector was operated with a glassy carbon electrode at 1.4 V, a $Ag/AgCl/Cl^-$ (3 M) reference electrode and a stainless steel auxiliary electrode. For voltammetric experiments, different working electrodes (Pt, Au, glassy carbon) were mounted in the flow cell of the Dionex PAD-2 detector, which was electrically connected to a polarographic analyzer, model 264A (EG and G PAR, Princeton, USA). Fluorimetric detection was achieved at $\lambda_{excitation}=365$ nm and $\lambda_{emission}=435$ nm, while spectrometric detection was at $\lambda=360$ nm. A WO Electronics Mod. BFO-04 oven was used to thermostat the column at 40°C. The chromatographic separation was performed on a PAH-16 Plus Bakerbond 25 cm×3.0 mm I.D. stainless steel column with 5-μm packing (Baker) using a methanol–acetonitrile–water (25:25:50, v/v/v) mobile phase at 0.7 ml/min. HPLC-grade solvents (Merck) and 0.1 *M* $LiClO_4$ (Merck) were used throughout the investigations. Purified water was obtained from a Seralpur PRO90CN system (Seral, Ransbach, Germany). A mixture of aflatoxins was purchased from Supelco (Bellefonte, PA, USA) (catalogue no. 4-6303), aflatoxins B_2 [CAS no. 7220-81-7] and G_1 [CAS no. 1165-39-5] were obtained from Aldrich (catalogue nos. 85,621-5 and 85,622-3, respectively).

2.2. Procedures

For the stability study, a Perimax (Spetec) Model 12 peristaltic pump was used at 20 rpm to circulate the aflatoxin solutions across the electrochemical cell in a 50 cm×1/16 in. I.D. PTFE loop connected to a 1.0-ml sample vial (1 in.=2.54 cm).

Another tubular knitted PTFE loop (4.5 m×1/16 in. I.D.) was immersed in a Magidigest (Prolabo, France) Model MX-350 microwave bath and connected to a sample vial. After different electrolysis-, radiation- or heating times, the vials were disconnected in both cases from the PTFE loop and 10 μl of the solution were injected. Both off-line and on-line UV irradiation at 254 nm was applied as the photo-derivatization procedure along the same 4.5 m×1/16 in. I.D. PTFE loop as used in the post-column reactor.

3. Results and discussion

Aflatoxin molecules exhibiting target functional groups and double bonds are able to undergo reduction/oxidation reactions, which could generate a measurable current when a sufficient potential is

applied. Fig. 1 shows cyclic voltammograms of aflatoxin G_1 using different electrodes in the same supporting electrolyte solution. In contrast to the bad electrochemical behavior on platinum and gold electrodes (Fig. 1B–C), on the voltammogram using the glassy carbon electrode, a high current difference was observed at a potential of about 1.2 V when the current of the supporting electrolyte was measured with and without aflatoxin G_1 (Fig. 1A). This shoulder, indicating oxidation of the molecule, provided the motivation for the employment of electrochemical detection in the HPLC analysis of the main group of mycotoxins. Assuming that the generated current is directly proportional to the concentration of the species under oxidation in the column effluent, we measured peak intensities at different applied potentials to examine the hydrodynamic voltammograms. Each curve was obtained by varying the potential from low to high values and then vice versa. Here, reproducible peak heights were obtained, except for B_2. As expected, current intensity increases with the applied potential (Fig. 2). The curves show, firstly, that reaction starts for aflatoxins B_2 and G_2 just at 1.2 V and, secondly, that electroactivity of the studied aflatoxins increases in the order $G_2<B_1<B_2<G_1$. This order reflects the ability of the aflatoxin molecules to undergo electrochemical oxidation. The electrochemical response is higher for G_1, which is the molecule containing a higher number of unsaturated bonds and a pyrone-like group. It can also be noted that, at a potential higher than 1.45 V, the current response remains almost constant. For this reason, this potential value was chosen for the chromatographic work, and it was kept constant during the separation of a standard aflatoxin mixture.

It is known that, when aflatoxin B_1 is detected in contaminated grains, the presence of B_2 is unavoid-

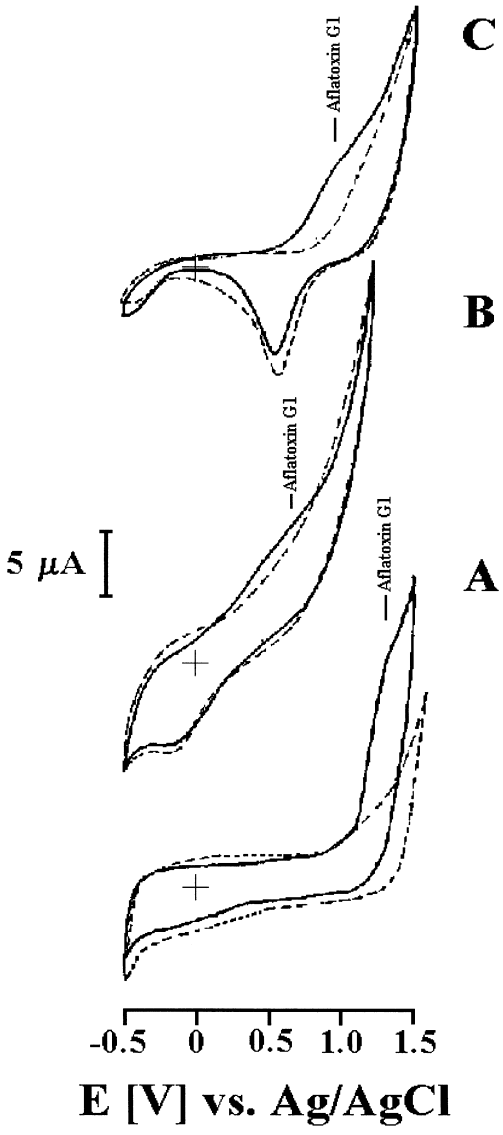

Fig. 1. Cyclic voltammograms of aflatoxin G_1 in a methanol–water (1:1, v/v) solution on different electrodes. A=Glassy carbon, B=Pt, C=Au. Dotted line, supporting electrolyte; solid line, supporting electrolyte with aflatoxin G_1. Concentration of aflatoxin G_1 ca. 0.6 mg/ml; supporting electrolyte, 0.05 *M* $LiClO_4$; scan rate, 100 mV/s.

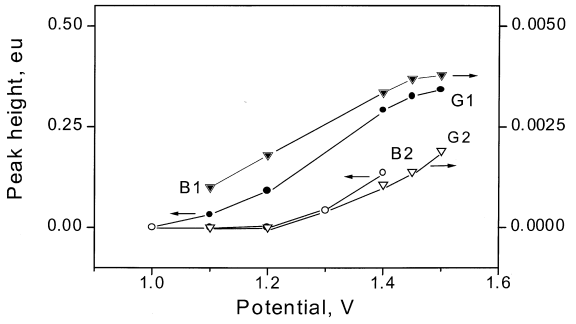

Fig. 2. Hydrodynamic voltammograms of aflatoxins in methanol–acetonitrile–water mobile phase using $LiClO_4$ as the supporting electrolyte.

able and aflatoxins type G can also be found. Since aflatoxin G_1 showed a good electrochemical response, it was selected together with aflatoxin B_2 for the rest of the study. This allowed for less hazardous work, evading the manipulation of aflatoxin B_1 and, on the other hand, we could concentrate our attention on the less resolved components in the RP-HPLC separation. The amperometric response was linearly related to the concentrations of both aflatoxins. The linear regression equations relating peak areas, *A*, with concentration, *C*, were $A=-43+3153C$ ($R^2=0.9975$) for aflatoxin G_1 and $A=-9+17C$ ($R^2=0.9924$) for aflatoxin B_2, showing an intercept that was not significantly different from zero.

Chromatograms of model mixtures of two and four aflatoxins with amperometric detection using a glassy carbon electrode at a potential of 1.4 V are presented in Fig. 3. It can be seen that aflatoxin G_1, specially exhibiting a lower fluorescence response, has an enhanced electrochemical response in comparison with aflatoxin B_2. The separation (Fig. 3, trace c) of four aflatoxins at concentrations of 0.3–1.0 μg/ml with electrochemical detection and 50 μl injection volumes indicates, as seen from the noise level, that the detection limit can be estimated at least in 10 ng for aflatoxins B_2 and G_2, and 7 ng for aflatoxins B_1 and G_1. This sensitivity can be compared with that obtained on a mercury electrode by reducing aflatoxins [14].

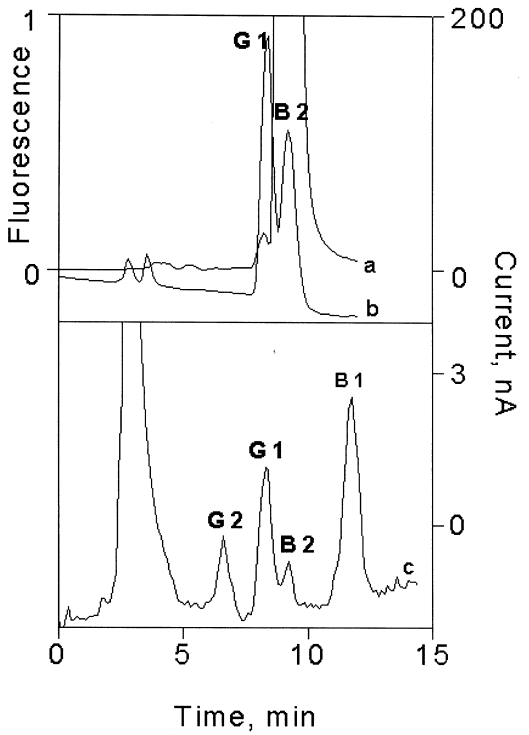

Fig. 3. Comparison of fluorescence (a) and amperometric electrochemical detection (b, c) of two aflatoxin mixtures. Concentrations of $1\cdot10^{-3}$ *M* for G1 and $4\cdot10^{-3}$ *M* for B_2 (a, b), $1\cdot10^{-6}$ *M* for G_2, B_2 (c) and $3\cdot10^{-7}$ *M* for G_1, B_1 (c). Injection volume, 50 μl. Chromatographic conditions were as described in Section 2.

The widespread occurrence of aflatoxins has lead to the introduction of measures to partially destroy them by the implementation of different practices. Methods of reducing aflatoxin content in feeds include radiation and heat treatments. The effect of electrolysis, UV irradiation and temperature on the decomposition of aflatoxins G_1 and B_2 was studied by carrying out an off-line procedure and afterwards measuring the peak area diminution of the corresponding signal. When electrolysis of aflatoxin G_1 as the model substance at 1.4 V was carried out off-line and analyzed after different time intervals, a high number of new peaks appeared in the chromatogram, indicating the formation of decomposition products that could be detected by their fluorescence response (Fig. 4). Decomposition kinetics were investigated for aflatoxins G_1 and B_2 by integrating the chromatographic peaks and normalizing the peak areas after electrolysis relative to the area of the initial peak. Fig. 5a shows that aflatoxin G_1 undergoes oxidation to a much greater extent than aflatoxin B_2,

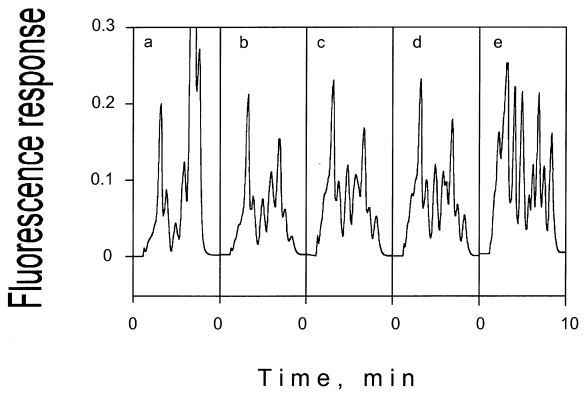

Fig. 4. Aflatoxins G_1 before (a) and after electrolysis at 1400 mV for 60 min (b), 90 min (c), 120 min (d) and 240 min (e). Chromatographic conditions were as described in Section 2.

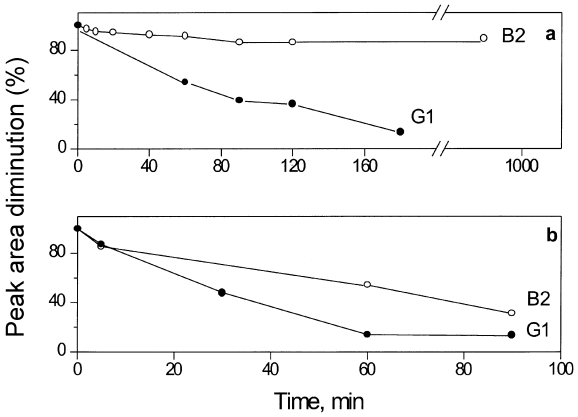

Fig. 5. Effect of electrolysis at 1.4 V (a) and UV irradiation (b) on the decomposition of aflatoxin B_2 and aflatoxin G_1. Decomposition is given as the peak area diminution by amperometric detection.

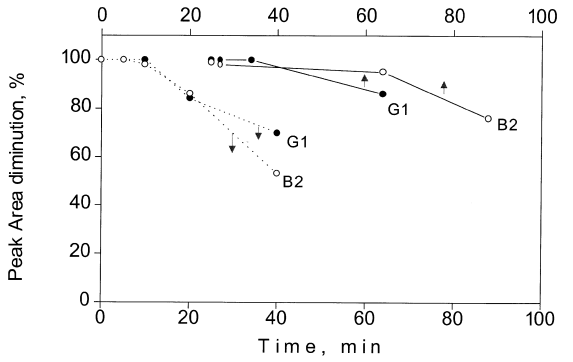

Fig. 6. Effect of heat treatment time at 100°C (dotted line) and of temperature on heating for 3 min (continuous line) on the decomposition of aflatoxins. Temperature was varied by changing the microwave bath power. Peak area diminution was measured by amperometric detection for G_1 and by fluorescence detection for B_2.

while off-line UV irradiation produces an intense effect on both compounds and occurs readily (Fig. 5b). For aflatoxin B_1, European Union collaborative studies have demonstrated that admittance of daylight into the laboratories caused concentration losses. In our study, diminution of the concentration of aflatoxin B_2 was slower than that of aflatoxin G_1, but approached the same residual value for both compounds. It is also marked that, after 60 min of radiation, aflatoxin G_1 suffers practically no more decomposition.

The influence of the treatment temperature on decomposition was studied by immersing the aflatoxin solution for 3 min in a microwave oven bath that had been warmed previously to a defined temperature. The effect of the duration of the heat treatment was followed by immersing the solution in a 100°C bath for different time intervals. The results indicate that, when the studied aflatoxin solutions were placed in a 90°C bath for 3 min, a maximal value of 20% peak area diminution was obtained for both G_1 and B_2 aflatoxins (Fig. 6, continuous line). A greater extent of decomposition (50%) is observed however, if the treatment at 100°C is maintained for longer time (Fig. 6, discontinuous line). Aflatoxin B_2 decomposes to a larger extent than aflatoxin G_1, indicating a lower stability against prolonged heat treatment.

Finally, the effect of post-column photo-derivatization on the amperometric response was studied. We succeeded in obtaining a moderate enhancement of peak heights for aflatoxins B_2 and G_1, which were not comparable with the reported [13] growth in the fluorescence response.

4. Conclusion

As a result of our work, a new amperometric detection method is recommended to detect and quantify the less toxic aflatoxin B_2, which is always present in grains contaminated with aflatoxins. Aflatoxin G_1, which has a smaller fluorescence response in UV detection and a 20% smaller extinction coefficient in comparison with aflatoxin B_1, has an amperometric detection limit of 7 ng using $LiClO_4$ as the electrolyte and a glassy carbon electrode at 1.4 V. The stability against electrolysis and UV irradiation is lower for aflatoxin G_1 in comparison with aflatoxin B_2, while G_1 is more resistant to heat treatment.

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