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Laser Fluorimetry: Detection of Aflatoxin B_1 in Contaminated Corn

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Aflatoxins, metabolites from Aspergillus fungi, are among the most potent naturally occurring carcinogens known.(1) The presence of these toxins in varying amounts in a wide variety of grain and food products in virtually every country of the world has given rise to concern over the potential public health hazard caused by aflatoxins in the human food supply.(2) The carcinogenic activity of these compounds at the low ppb level, documented in feeding experiments with laboratory animals, (1) ipso facto demands analytical detection methods for aflatoxins at this level or better. From the feeding experiments, the most pronounced effects of aflatoxin, both accute and chronic, are known to occur in the liver. In trout fry, aflatoxin has been shown to induce hepatoma in statistically significant percentages at a level of only 100 ppt (1)--a disquieting fact given that conventional analytical techniques are generally capable of detecting aflatoxin at ten to a hundred times this concentration.

By far, the most widely used method for aflatoxin detection relies upon separation of the aflatoxins on a TLC plate followed by visual observation of their natural fluorescence when excited by a UV lamp. Depending upon the skill of the individual experimenter, this method has a detection limit from 1 to 10 ppb in corn. Attempts to improve upon this procedure by forming an extract from a larger grain sample, or equivalently by spotting a larger amount of grain extract on the TLC plate are of little value since overloading of the TLC plate results, i.e. band spreading of various components in the extract occurs in the region of aflatoxin fluorescence, precluding more sensitive detection.

Taking advantage of the partial cleanup of the extract afforded by an overloaded TLC plate, we report here a two-step chromatography procedure, preparative TLC followed by reverse phase HPLC, for quantitation of aflatoxin B_1 in corn. Detection

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of aflatoxin B_{2A} (formed from aflatoxin B_1) is carried out with a high sensitivity laser fluorimeter that can detect as little as 750 fg of aflatoxin. The procedure outlined is capable of linearly quantitating aflatoxin B_1 from 0.1 to 10 ppb in white and yellow corn.

Apparatus

The laser fluorimeter (3) is based on phase sensitive detection of fluorescence excited by an amplitude modulated, 8 mW, He-Cd ion laser. The normal drift in output power of the laser is virtually eliminated by a feedback loop consisting of an acousto-optic light modulator, difference amplifier, and photodiode employed in the Liconix Model 405 UV laser. As shown in Fig. 1, an oscillator drives the difference amplifier at 50 kHz, which by virtue of the feedback loop, produces a 100% amplitude modulated beam directly in phase with the oscillator. After passing through a UV pass filter (Corning 7-60) to remove background radiation from the plasma tube, the beam is focused by a quartz lens into a flowing liquid droplet of eluent from the HPLC column. By positioning the collimator so that the surface of the droplet where the laser enters is excluded from view, scattering of the laser radiation into the detection optics is minimized. Fluorescence excited in the droplet passes through an interference filter, a quartz lens, and a visible pass filter located in a separate polished brass chamber. The first filter after the collimator is a front surface, long wavelength pass interference filter on a spectrosil quartz substrate. The 1.25 in. focal length quartz lens focuses light from the droplet onto the photocathode of a low noise photomultiplier (Centronix 4249BA). The photomultiplier signal is fed into a lock-in amplifier and detected in phase with the oscillator signal. The output of the lock-in amplifier, which is proportional to the fluorescence intensity from the droplet, is displayed on a stripchart recorder.

The fluorescence cell is a liquid droplet of eluent in the shape of Plateau's unduloid (4) which is supported by surface tension in a gap between the 1/16 in. O.D. tubing from the chromatography column and a solid stainless steel rod of the same diameter. The droplet forms a detection cell with a volume of only 4 μl and yet does not suffer from problems of cell wall fluorescence. Bubbles are prevented from entering the droplet by notching the tubing near the end, and plugging the tip so that the eluent is forced to flow down the sides of the tubing allowing bubbles to rise to the surface. After passing down the outside of the stainless steel rod, the eluent is removed by an aspirator. Some scattering of the beam by the droplet cannot be avoided. To minimize the resulting fluorescence from the light shield, a coating of fine carbon (Fisher Norit A in chloroform) was applied to the interior surfaces of the light shield.

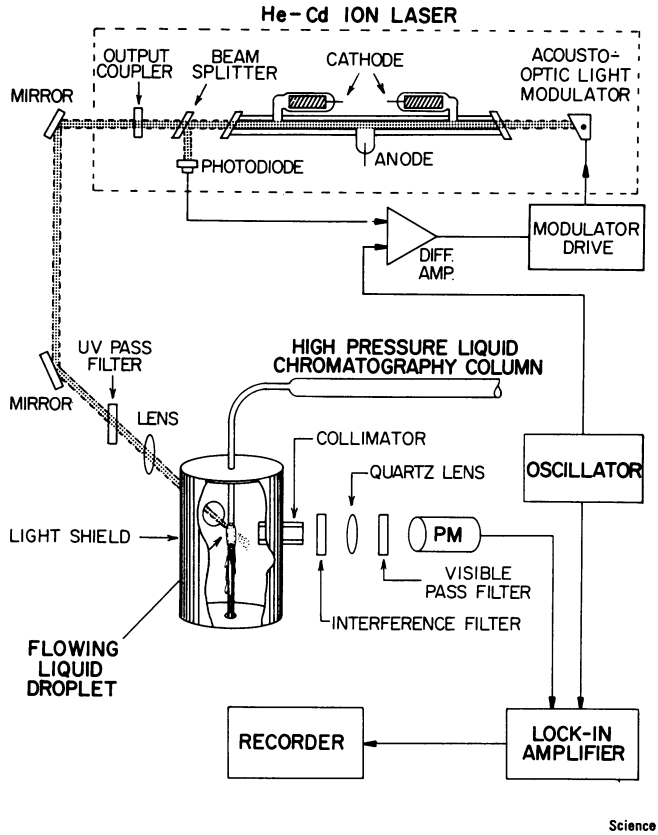

Figure 1. Diagram of the feedback stabilized laser, flowing droplet fluorescence cell, and detection electronics (3)

The sensitivity of this device for aflatoxin detection is demonstrated in the chromatograms shown in Fig. 2. Here, the aflatoxins B_1 and G_1 have been derivatized to the more fluorescent aflatoxins B_{2A} and G_{2A}.

As a result of the partitioning processes in the HPLC column, the aflatoxins elute from the column considerably more dilute than the solution injected onto the column; thus, the detector must respond to a correspondingly less fluorescent solution. The peak eluting from the column has a Gaussian profile given by $f(x) = (2\pi)^{-\frac{1}{2}} \sigma^{-1} \exp(-x^2/2\sigma^2)$ where $3.56\ \sigma$ is the full width at half maximum intensity of the peak. A typical peak has a full width at half maximum of 0.4 min which corresponds to $\sigma = 0.17$ ml at a flow rate of 1.5 ml/min. Since $f(x)$ is normalized to unity, the concentration of aflatoxin at the detection limit (Fig.2) becomes $c(x) = 750\ f(x)$ in fg/ml. At the maximum in the peak, $c(0) = 1800$ fg/ml corresponding to 6×10^{-12}M. Since the detection volume is only 4 µl, the amount of aflatoxin in the droplet is only 7 fg corresponding to 1×10^7 molecules.

Aflatoxin B_1 Detection in Contaminated Corn

Extraction of aflatoxin from corn is carried out using the method of Seitz and Mohr.(5) The final extract represents 10 g of grain and is dissolved in 0.5 ml of a benzene-acetonitrile solution. The first cleanup step consists of spotting 50 µl of this extract onto a Brinkman SIL-G-25-HR TLC plate which is developed in an 88:12 v/v chlororform-acetone solution. Now, even at 1 ppb, the 50 µl of extract corresponds to only 1 ng of aflatoxin--an amount difficult to identify on a TLC plate. To provide certain identification of the position of the aflatoxins in the unknown samples, 10 ng standards of aflatoxin B_1 are spotted adjacent to the unknowns. Following development of the plate, the portion of the silica gel containing the unknown sample can be easily found by noting the position of the 10 ng standards under a UV lamp. The silica gel containing the aflatoxin is then removed from the TLC plate, agitated several minutes in a vial with 2 ml of chloroform and the supernatant collected. The sample is evaporated to dryness under a stream of nitrogen on a steam bath and the aflatoxin B_1 converted to aflatoxin B_{2A}, (1) known to have a high fluorescent quantum efficiency in hydrogen bonded solvents.(6) Although trifluoroacetic acid has been used, (7) we prefer to use 100 µl of 1N HCl (8) which is allowed to react for 10 min. The acid is then evaporated under nitrogen and the extract dissolved in 100 µl of the HPLC elution solvent.

The HPLC column used in these experiments is a Waters Inc. C18 µBondapak, reverse phase column operated at 1.5 ml/min with a 75:25 water-ethanol solution. Solvents are of extremely high purity. Ethanol is prepared (9) by distilling 95% "gold shield"

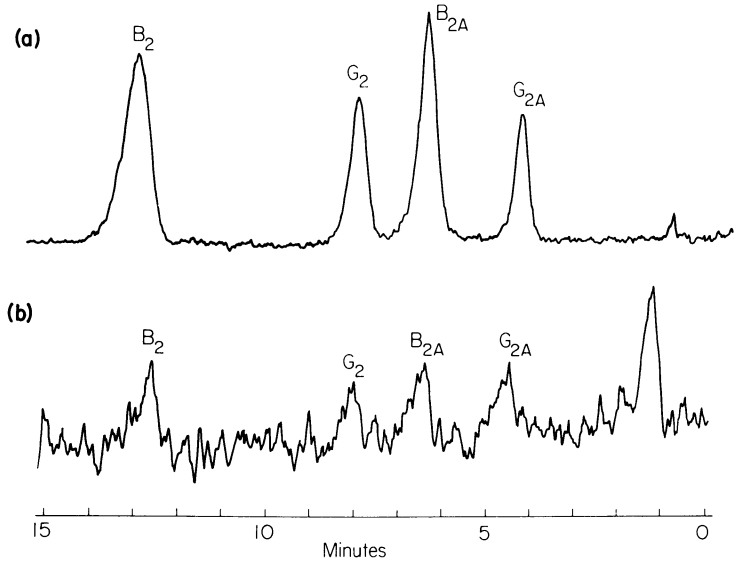

Figure 2. (a) Chromatogram of aflatoxins B_1, G_1, B_2, and G_2 eluting from a C18 μBondapak HPLC column at a flow rate of 1.5 mL/min; 30 pg each. The aflatoxins B_1 and G_1 have been converted to aflatoxins B_{2A} and G_{2A}, respectively, with HCl. (b) The fluorimeter response at the detection limit of 750 fg. A three-second time constant was used in (b); at higher levels a one-second or shorter time constant on the lock-in amplifier can be used (3).

ethanol to which 1 g KOH pellets per liter has been added. After discarding the first few ml of distillate, approximately 2/3 of the original volume is collected. Water is filtered, deionized, passed over activated charcoal, and distilled. After mixing, solvents are degassed in an ultrasonic cleaner for 1/2 hour. Great care must be taken to insure that all surfaces coming into contact with the solvents are free of contamination. Thorough cleaning with a 10% HF solution is recommended. The purity of the solvents can be given a final test by visually observing fluorescence excited by the laser. In a completely darkened room, fluorescence over a few cm path length should be faintly visible, or not visible at all. The column also must be free of contamination. Passing high purity ethanol through the column, as recommended by the manufacturer, is effective in removing various fluorescent contaminants from the column.

After a stable baseline is obtained on the recorder, 10 µl of the extract are injected onto the column. The fluorimeter sensitivity, and column retention time for aflatoxin B_{2A} are determined by preparation of known quantities of aflatoxin B_1 which are spotted on TLC plates, derivatized, etc. in the same manner as the unknown sample. The aflatoxin content of the contaminated grain is determined by comparing the peak in the unknown sample to those from standards. If the above procedure is followed exactly, 1 ppb of aflatoxin contamination corresponds to 100 pg of a standard injected directly onto the HPLC column.

Results

In a previous paper, (3) the linear response of the detector was demonstrated over the range from 750 fg to 30 ng for aflatoxins B_{2A}, G_{2A}, B_2 and G_2. The linearity of this method for aflatoxin quantitation rests on a constant conversion of aflatoxin B_1 to B_{2A}, and therefore the conversion efficiency, although reported to be high, (8) must be scrutinized over the entire range of interest. Thus, aflatoxin B_1 standards ranging from 10 pg to 5 ng were individually derivatized, redissolved in elution solvent, and injected onto the HPLC column. The magnitudes of the fluorescence signals, as shown in Fig. 3, exhibit the expected linearity over roughly three orders of magnitude. The straight lines are least squares fits to the data determined by minimizing the fractional error between the data points and a line through the origin. (This procedure weights each point equally, whereas a conventional least squares fit strongly favors data points with large values.) The root mean square (rms) error was then calculated for each run giving an average of 28% for the two runs.

The overall precision of this method is determined by errors in recovery from the TLC plate, derivatization, and quantitation in the fluorimeter. Further errors introduced in the extraction procedure have been previously investigated.(5) Standards of aflatoxin B_1 were spotted on TLC plates, recovered, derivatized, and injected onto the HPLC column. The results shown in Fig. 4 surprisingly show a marked departure from linearity above 1 ng indicating an increased recovery from the TLC plates. Since this change in recovery is not particularly pronounced, standards could be used to calibrate the procedure permitting accurate quantitation at any level of aflatoxin contamination from 0.1 to 50 ppb (10 pg to 50 ng). However, since the simpler method of fluorimetric quantitation directly on TLC plates can be used above the 10 ppb level it appears prudent to limit the range of the HPLC-laser fluorimetric method to levels below 10 ppb, thus simplifying the error analysis. With this constraint an rms error of 26% (an average of both runs in Fig. 4) obtains for the range between 0.1 and 10 ppb.

The efficacy of the two-step chromatography procedure for eliminating interfering components in the corn extract is shown in Fig. 5a. The smoothness of the baseline can be compared to the signal in Fig. 5b, representing a contamination of 250 ppt, thus indicating a limit of detection for this method of approximately 100 ppt. Note that since the detection limit is determined by the concentration of contaminants still remaining in the final extract, higher fluorimeter sensitivity (below 10 pg) would not further improve detection limits. Thus a reduction in the above figure for detection of aflatoxin in corn is contingent upon the development of either more specific detectors, or further improvements in cleanup techniques.

Discussion

An inherent advantage of this technique lies in the ability to use the derivatization step as a confirmatory procedure for the presence of aflatoxin B_1. Since aflatoxin B_1 has a low fluorescent quantum efficiency in solution, comparison of chromatograms where the derivatization step has been deleted provides additional evidence of its presence in the grain sample.

The ultimate sensitivity of the fluorimeter described here is contingent upon a fortuitous coincidence between the laser line at 325 nm and an absorption maximum of the species of interest. However, in practice remarkable sensitivity is attainable even when this condition is not fulfilled. Fluorescein, for instance, has an absorption maximum at 500 nm, a considerable shift from the laser line. While the optical density at 325 nm differs from that at the peak by four, (10) corresponding to a factor of 10^4 in absorption, the fluorimeter

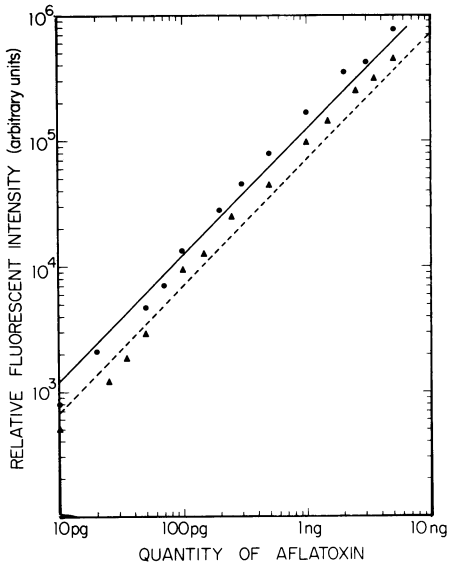

Figure 3. The fluorimeter signal, proportional to the amount of aflatoxin B_{2A}, is plotted as a function of the quantity of aflatoxin B_1 derivatized to determine the linearity of the HCl-derivative formation procedure. The displacement of the two lines on the log–log plot corresponds to slightly different fluorimeter gains for the two runs. (– ● –) Run 1. (-- ▲ --) Run 2.

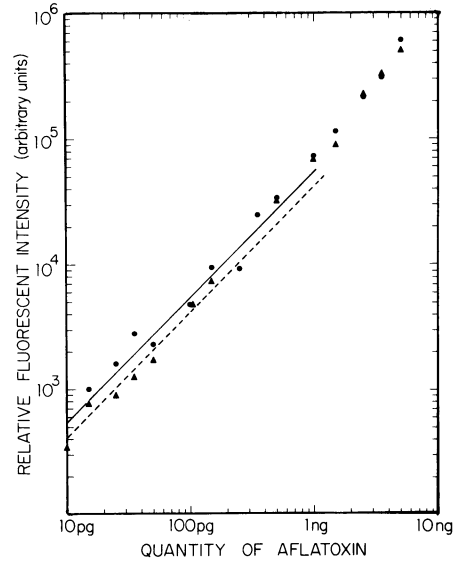

Figure 4. Aflatoxin B_1 standards were spotted on TLC plates, removed, derivatized, and quantitated with the fluorimeter. The fluorimeter response, plotted on the ordinate, shows a slight nonlinearity above 1 ng. (– ● –) Run 1. (-- ▲ --) Run 2.

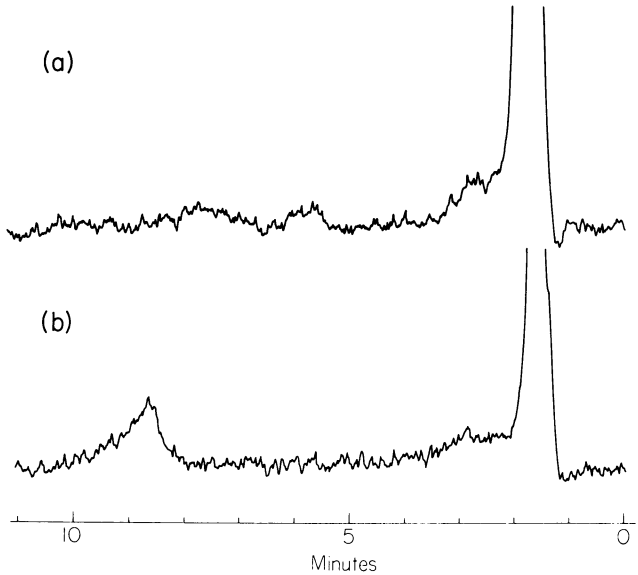

Figure 5. (a) Chromatogram of an aflatoxin-free sample of yellow corn using the cleanup procedure described in the text. The flatness of the baseline compared with the signal from the 25 pg aflatoxin B_{2A} (B_1) standard in (b) indicates a sensitivity of approximately 100 ppt. The tailing seen in these chromatograms is attributable to deterioration in the two-year-old column. The sharper peaks expected from a new column would suggest a slightly improved sensitivity.

is still capable nevertheless of detecting 4 pg of this dye injected onto the HPLC column. In the case of the aflatoxins, absorption maxima lie at 360 nm, yet the detection limit of this device is 750 fg of each of the four aflatoxins injected onto the HPLC column.

We believe that laser fluorimetry is applicable to a number of trace analysis problems, and has special advantage in those cases where sensitivity is a limiting factor.

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Abstract

A high sensitivity laser fluorimeter designed specifically for use with high pressure liquid chromatography (HPLC) is described. An amplitude modulated He-Cd ion laser at 325 nm irradiates a flowing droplet of eluent from an HPLC column, and fluorescence is detected in phase with the modulation by a lock-in amplifier. The fluorimeter is capable of detecting 750 fg of each of the four commonly occurring aflatoxins, and has a linear range of over three orders of magnitude. Using a two-step chromatography procedure, this device is capable of quantitating aflatoxin B_1 to 100 ppt in corn. Following extraction of the aflatoxin from corn, the extract is given a preliminary cleanup on a normal phase thin layer chromatography (TLC) plate. The aflatoxin B_1 is recovered, and injected onto a reverse phase HPLC column. Experiments with aflatoxin B_1 standards show a constant ratio for conversion of aflatoxin B_1 to aflatoxin B_{2A} over roughly three orders of magnitude. The recovery of aflatoxin B_1 from the TLC plates, although slightly nonlinear above 10 ppb permits linear quantitation of aflatoxin B_1 in white and yellow corn in the range from 0.1 to 10 ppb with an average root mean square (rms) error of 26%.

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