Determination of Zearalenone in Corn by Laser Fluorimetry

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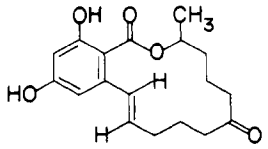
By combining laser fluorimetry with high pressure liquid chromatography, we are able to detect and quantitate the naturally fluorescent mycotoxin, zearalenone, in contaminated corn samples. Experiments with zearalenone standards show a linear fluorimeter response over four orders of magnitude with a detection limit of 300 pg zearalenone injected onto a C_{18} reverse phase column. The corn samples are first purified using a small silica gel column. The recovery from this step is 86% over the range from 5 ppb to 2.5 ppm. Based on the magnitude of the zearalenone signals compared to the flatness of the base line for zearalenone-free corn samples, a limit of 5 ppb is placed on the detection of zearalenone by this procedure.

In previous studies, we have illustrated the advantages of laser fluorescence as a detector of trace species in thin-layer chromatography (TLC) and high pressure liquid chromatography (HPLC) by applying this technique to the quantitative analysis of aflatoxins (*1–4*). The present work reports the use of laser induced fluorescence detection combined with HPLC separation for the analysis of zearalenone, also known as F-2 toxin. Zearalenone (6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)-β-resorcylic acid μ-lactone



is a potent estrogenic mycotoxin suspected of causing infertility in dairy cattle and swine through the ingestion of moldy feedstuff (*5, 6*). Derivatives of zearalenone have been used by the livestock industry as an anabolic agent to promote growth. While its toxicity appears to be only moderate in comparison to several other mycotoxins, a rather high incidence of zearalenone contamination has been documented in several published reports (*5, 7*), giving rise to concern about the possible contamination of the human food supply.

Zearalenone and its derivatives exhibit fluorescence when irradiated by UV light. Irradiation of zearalenone developed on a TLC plate with long wavelength UV (356 nm) causes blue-green fluorescence, while irradiation at short wavelength (254 nm) causes a more intense fluorescence (*5*). These fluorescence properties have served as the basis for detecting zearalenone. In particular, rapid screening of grain samples using TLC separation with fluorescence detection has achieved sensitivities of several hundred ppb (μg/kg) (*8–11*), while one TLC study reports a detection limit as low as 50 ppb (*12*). These detection limits might be decreased further by the use of a special reagent for the development of TLC plates spotted with zearalenone (*13*). The superior resolving power attainable with HPLC has been applied to zearalenone determination (*14, 15*). Using UV absorption at 254 nm, a lower detection limit of 5 ng has been achieved by Holder, Nony, and Bowman (*14*); using a many-step clean-up procedure, the same authors reported a 10-ppb detection limit for zearalenone in animal chow. Recently an HPLC method using UV absorption detection at 236 nm has been reported by Möller and Josefsson (*16*) with a detection limit of 600 pg for zearalenone standards, and 2 ppb for several contaminated grains.

Here we report laser fluorescence detection of zearalenone eluting from an HPLC column. The laser fluorimeter shows a linear response to zearalenone standards injected onto the column from 300 pg to 3 μg. We use a short silica gel column for cleanup followed by separation with reverse-phase HPLC. This procedure gives sufficient resolution between zearalenone and other contaminants so that when this is combined with the high sensitivity of the laser fluorimeter, quantitation of zearalenone in contaminated corn is achieved to 5 ppb.

EXPERIMENTAL

The laser fluorimeter attached to the HPLC system has been described previously (*2–4*). The eluate from a reverse-phase HPLC column passes through a flowing liquid droplet, suspended between two pieces of stainless steel tubing. This droplet forms a windowless fluorescence cell which is irradiated by the 325-nm output of a He-Cd ion laser. The laser is amplitude modulated at 55 kHz and fluorescence is detected by a photomultiplier whose output feeds a lock-in amplifier. The output from the lock-in amplifier is displayed on a stripchart recorder.

The zearalenone extraction procedure is a slightly modified version of the Seitz and Mohr method for quantitation of aflatoxin in corn (*17*). Briefly, a 50-g sample of ground corn is extracted twice with water–methanol (25 + 75, v/v). Ammonium sulfate is added to a portion of the initial extract and the aqueous phase partitioned with hexane. The hexane extract is washed twice with water–methanol (25 + 75, v/v), and the water–methanol washings are added back to the original aqueous phase. The aqueous phase is then partitioned twice with methylene chloride to extract the zearalenone. The methylene chloride is evaporated under nitrogen on a steam bath and the residue, representing 10 g of grain, is dissolved in 0.5 mL of benzene–acetonitrile (98 + 2, v/v). Thus, the extraction procedure is identical to the method described in ref. 17 with the exception of the additional washing step for the hexane.

Before the extract is cleaned up on a silica gel column (Waters Inc., Sep-Pak), 35 mL of diethylether–hexane (75 + 25, v/v) are passed through the column to remove impurities from the silica gel column that were found to have the same retention time as zearalenone in HPLC. The corn extract is redissolved in an equal volume of high purity ethanol by evaporation under nitrogen on a steam bath, and 100 μL are placed on the silica gel column. The zearalenone is eluted from the column with 2.5 mL of the ether–hexane solution which is evaporated to dryness as before and redissolved in 100 μL of ethanol.

After a stable base line is obtained on the stripchart recorder, 10 μL of the purified extract are injected onto the HPLC column. The C_{18} Bondapak column (Waters Inc.) is operated at a flow rate of 1 mL/min with a (50 + 50, v/v) high purity water–ethanol solution. Failure to observe the stringent purity precautions described previously (*2–4*), will mitigate against high sensitivity detection.

The concentration of zearalenone in the corn sample is determined by comparison with zearalenone standards. If the above procedure is followed, 1 ng of a standard injected directly onto the column corresponds to 5 ppb zearalenone contamination of the grain, assuming 100% recovery from the silica gel column. In practice, we find the recovery is slightly less than this figure

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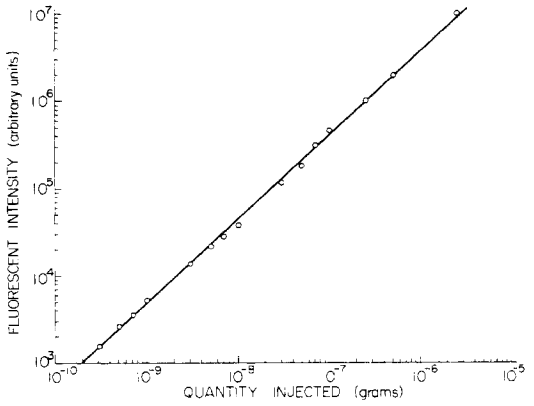

Figure 1. Fluorimeter response as a function of the quantity of zearalenone standard injected onto the HPLC column

(see below), introducing a small correction.

RESULTS

Figure 1 shows the response of the fluorimeter to various quantities of zearalenone standards injected onto the HPLC column. The detection limit for zearalenone is 300 pg; at this point the signal-to-noise ratio was approximately 2 to 1. The fluorimeter response is seen to be linear over four orders of magnitude. The data point at 3 μg corresponds to 15 ppm. The response linearity suggests that this method might be used to quantitate zearalenone at still higher levels of concentration.

Recovery of zearalenone from the silica gel column was investigated by spotting 100-μL samples of appropriately diluted zearalenone standards on the column. Following elution (as described above), the standards were redissolved in the ethanol and a 10-μL portion was injected onto the HPLC column and quantitated in the fluorimeter. The amounts of zearalenone recovered from the silica gel column are compared to standards injected directly onto the column. Over the range from 1 to 500 ng, we find that the recovery of zearalenone from the silica gel column is relatively high and constant, namely, 86.2% with a standard deviation of 3.4%.

A typical chromatogram of a zearalenone standard is shown in Figure 2a. The elution time, which can be varied by changing the relative concentrations of water and ethanol, was adjusted to provide the best separation between the zearalenone peak and various unidentified fluorescent compounds still present in the final extract. Figure 2b shows a chromatogram of a zearalenone-free corn extract; the flat base line is typical of a number of different corn samples that were studied. Fungal invasion of the corn did not result in any additional interference relative to uncontaminated corn in the region of the chromatogram where zearalenone elutes. The efficacy of the overall procedure for separating fluorescent compounds in the corn from zearalenone is evidenced by the flatness of the base line shown in Figure 2b. The flatness of that portion of the base line where zearalenone elutes determines the sensitivity of the method under the experimental conditions here, that is, under conditions where the limiting sensitivity of the detector has not been reached. Based on the relative response of the detector to zearalenone standards and zearalenone-free extracts, combined with our observation of easily recognizable zearalenone peaks from several spiked corn extracts, we estimate the detection limit for this method to be 5 ppb. A typical chromatogram showing the fluorimeter response to a 50-ppb contaminated yellow corn sample is shown in Figure 3.

DISCUSSION

In evaluating the performance of the laser fluorimeter, it is important to note that the 325-nm output of the He-Cd ion

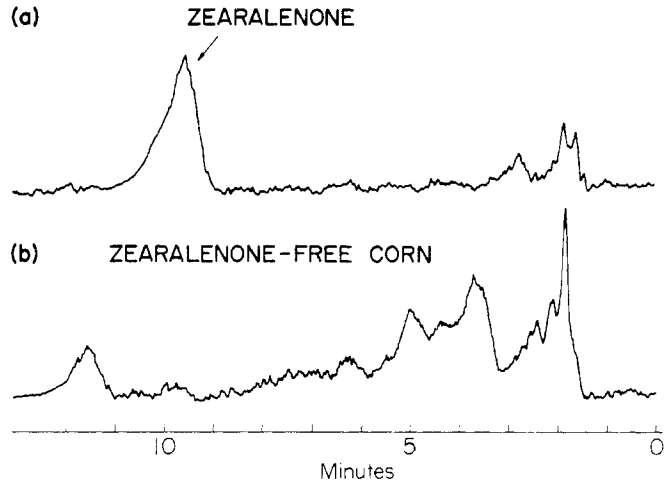

Figure 2. Chromatograms of (a) a 5-ng zearalenone standard and (b) a zearalenone-free corn extract, taken on the same gain scale. Zearalenone elutes at 9.6 min; the peak eluting after 11.6 min in (b) corresponds to an unknown impurity in the ether–hexane solvent used in the silica gel column. The corn extract was from a sample on which fungi were allowed to grow so as to be more representative of the problems encountered in analyzing moldy grain for the presence of zearalenone

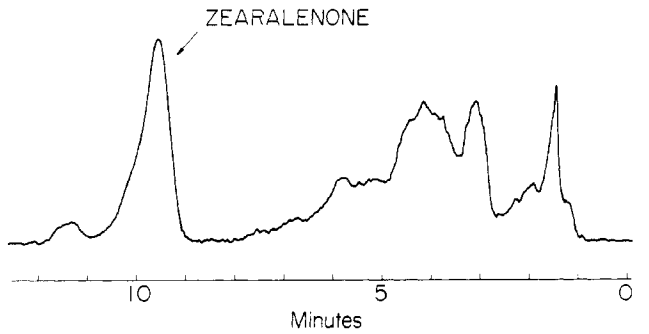

Figure 3. Chromatogram of a corn extract contaminated with 50 ppb of zearalenone standard. The peak eluting after 9.6 min is from zearalenone

laser excites zearalenone far from its absorption maximum (*16*) at 236 nm. In addition, zearalenone has a comparatively low fluorescent quantum efficiency (*18*). While the detection limit reported here for zearalenone standards compares favorably with those reported using UV absorption, clearly these two factors greatly diminish the sensitivity of the present technique. Previous applications of laser fluorimetry for aflatoxin detection (*2–4*) have proved more fruitful in terms of both detection limits for standards as well as for quantitation of aflatoxin in grain at low levels of contamination. The difference in these two cases arises mainly as a result of the higher fluorescent quantum efficiency of aflatoxin (*18*) at the excitation wavelength employed. If shorter wavelength lasers are used as the excitation source, one would anticipate a marked increase in the sensitivity of the laser fluorimeter to the detection of zearalenone.

It is instructive to compare the fluorescence detection described here with UV absorption detection of zearalenone presented in ref. 14 and 16. The detection limit for zearalenone standards reported here, 300 pg, is superior to the limit given in ref. 16 by a factor of two, and over an order of magnitude lower than the value reported in ref. 14. For grain samples, on the other hand, the detection limit for zearalenone contamination in terms of ppb reported here using fluorescence detection is almost identical to those reported using UV absorption detection. The comparative simplicity of the clean-up procedure given here as contrasted to the many-step

method described in ref. 16 (or in ref. 14 if any major differences between corn and chow are discounted) would appear to indicate that fluorescence detection possesses a higher specificity than UV detection for the problem at hand. A comparative study of the two detection methods using the same extraction and clean-up procedures would provide a definitive answer to this question.

However, from the point of view of carrying out routine laboratory analyses of grain samples, selection of one method over another is often largely a matter of personal convenience—even in those cases where fluorescence detection is significantly more sensitive for detection of standards. Since the size of the grain sample from which the extract is made can be arbitrarily large (on the scale of these analyses), detector sensitivity can be traded off against the effectiveness of the clean-up procedure: an increase in the sample size combined with a correspondingly more thorough cleanup will offset a loss in detector sensitivity without a sacrifice in the overall ability of the method to detect contamination in grain (in terms of ppb). An examination of the equivalent amounts of grain injected onto the HPLC column reported in this work (0.2 g) and the two papers describing UV detection of zearalenone in grain (1 g in ref. 14, 5 g in ref. 16) clearly demonstrates this tradeoff and points up the options open to the analyst in detecting contamination of grain.

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(18) Compare the absorbance reported for aflatoxin by J. A. Robertson, W. A. Pons, Jr., and L. A. Goldblatt, *J. Agric. Food Chem.*, **15**, 798 (1967) with that for zearalenone reported in reference 12. While the maximum absorbances are approximately the same, the detection limit on TLC plates for zearalenone (see reference 14) is about two orders of magnitude less than that for aflatoxin (see reference 1).

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Silicon Emitter for Field Desorption Mass Spectrometry

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A new ion emitter for field ionization and desorption mass spectrometry has been developed. Very fine silicon whiskers grown on a 60-μm (diameter) tungsten (or tantalum) wire can be used as a strong and efficient emitter. The process of growth is as follows. A small amount of gold is deposited by evaporation on 60-μm tungsten wire. After being preheated in a vacuum chamber, the gold coated tungsten wire is heated by direct electric current in silane gas (SiH_4 5% + Ar 95%) at a pressure of 180 Torr. Silicon whiskers grow in about 1 min. Pre-treatment, careful control, and high voltage supply during the whisker growth are unnecessary. The total ionizing efficiencies of silicon emitters in FI operation and in FD operation have been measured. The ionization efficiency for acetone in FI operation was 5×10^{-6} A/Torr and that for AMP in FD operation was 1.4×10^{-12} C/μg. The results for other test samples are also described.

Significant progress has been made in field desorption mass spectrometry (FD-MS) since Beckey first introduced it in 1969 (*1*). One of the most important problems in FD-MS is to obtain good FD emitters. Essential requirements of FD emitters are pointed out in Ref. 2 as: (1) high ionization efficiency, (2) large surface area, and (3) sufficient strength. The high-temperature activated carbon emitter is one solution that is now widely adopted as a standard emitter (*3–5*). Nickel and cobalt emitters (*6, 7*) also have recently been used in some cases. It seems to be desirable to add one more requirement which is, (4) easy production. A silicon emitter shortly reported in Ref. 8 would be a possible solution which satisfies the above four requirements. In our early work, silicon whiskers were grown on the 64-μm tantalum wire in silane gas (at 50 Torr) within about 15 min. After this initial work, systematic studies were done to obtain better silicon emitters. The detailed technique of producing silicon emitters and the ionization efficiency measurement of silicon emitters for some test samples (oligopeptides, nucleotides, organic salts) are described in this paper.

EXPERIMENTAL

Silicon Whisker Growing System. The apparatus is shown schematically in Figure 1. The growing processes are as follows. A 60-μm tungsten wire is spot welded on the wire support (1-mm covar wire). Gold is deposited by evaporation on the part of

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