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Detection of zearalenone in cereal extracts using high-performance liquid chromatography with post-column derivatization

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ABSTRACT

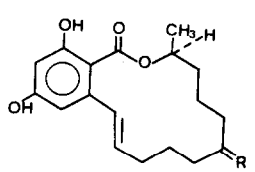
Post-column derivatization has been used to enhance the fluorescence response of the *Fusarium* mycotoxins zearalenone and zearalenol when determined by reversed-phase high-performance liquid chromatography. Derivatization is based on reaction with aluminium chloride and this results in a more selective response for these toxins. The method was tested on a number of cereals and animal feeds.

INTRODUCTION

Zearalenone is a fungal metabolite produced by a number of *Fusarium* species, and was originally isolated from *Fusarium roseum* and *Fusarium graminearum* [1]. Although not acutely toxic, zearalenone has been found to produce oestrogenic effects in animals and is suspected of causing infertility problems, especially in pigs [2]. Zearalenol is a structurally related *Fusarium* metabolite and produces similar oestrogenic effects [3]. The structural formulae for zearalenone and zearalenol are illustrated in Fig. 1.

A number of analytical methods have been developed for the determination of zearalenone and zearalenol in animal feedstuffs and human foods. Thin-layer chromatography (TLC) has been used for determination of zearalenone alone [4] or in multi-mycotoxin screening procedures [5,6]. However, the detection limits for zearalenone are relatively high, as it has a weak native fluorescence under UV light and, in order to improve the sensitivity of TLC, a number of reagents have been used to produce coloured spots when viewed under daylight or UV light. These include sulphuric acid [4], potassium hexacyanoferrate(III) and iron(III) chloride [4], bis-diazotized benzidine [7], aluminium chloride [5] and Fast Violet B [8].

More recently, high-performance liquid chromatographic (HPLC) methods have been developed for the determination of zearalenone, but owing to interference from co-extracted substances, extensive clean-up of the sample extracts is necessary prior to HPLC analysis to take full advantage of the sensitivity of modern detectors. This requires lengthy and time-consuming clean-up techniques such as liquid–



Name	R
Zearalenone	=O
Zearalenol	-OH

Fig. 1. Structural formulae of zearalenone (F-2) and zearalenol (ZAL).

liquid partition together with silica gel column chromatography to achieve detection levels as low as 10 μg/kg [8–10]. Even after clean-up using these methods, the final extracts produced are often unsatisfactory for the determination of zearalenone at this level. Hence any technique which improves selectivity and sensitivity would be advantageous.

Gas chromatography–mass spectrometry (GC–MS) has also been used and, when operated in the selected ion monitoring (SIM) mode, detection of zearalenone is both sensitive and selective [8]. A further development is the use of GC–tandem MS (GC–MS–MS), where multiple reaction monitoring (MRM) of daughter ions offers a highly specific detection method for zearalenone [11], but this type of instrumentation is expensive and may not be available to many laboratories. Recently, immunoassays have been developed for the determination of zearalenone, and an enzyme-linked immunosorbent assay (ELISA) method has been published for the determination of zearalenone in corn [12]. However these methods will require further evaluation before general acceptance for routine use.

A post-column reaction using aqueous iodine to enhance the fluorescence of aflatoxins B_1 and G_1 is now commonly utilized in aflatoxin analysis, when analysed by reversed-phase HPLC [13]. Aluminium chloride is known to enhance the fluorescence of zearalenone on TLC plates [5], and this reaction has been applied to the determination of zearalenone and zearalenol in sample extracts using the apparatus developed for the post-column derivatization of aflatoxins.

This on-stream reaction has been found to enhance the fluorescence of zearalenone and zearalenol by a factor of five without increasing the background signals from sample co-extractives.

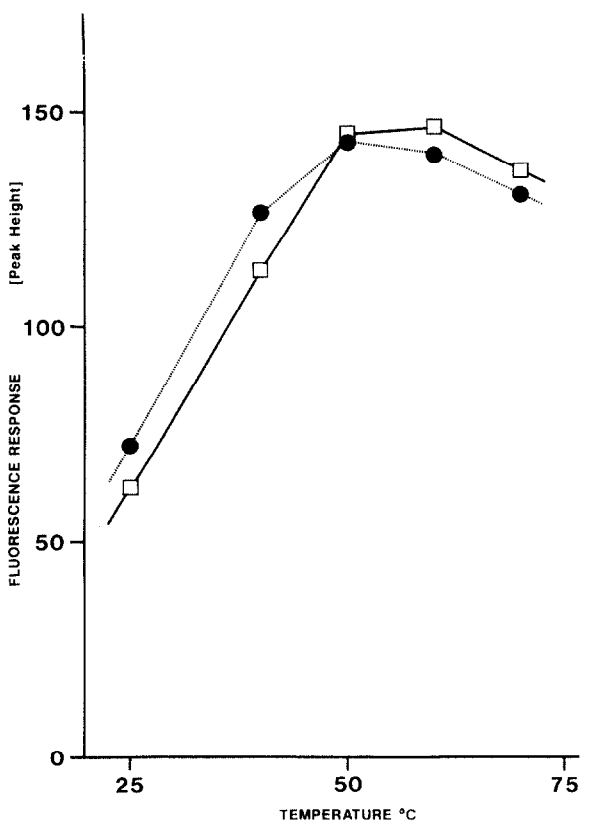


Fig. 2. Effect of temperature on the post-column derivatization of (□) zearalenone and (●) zearalenol.

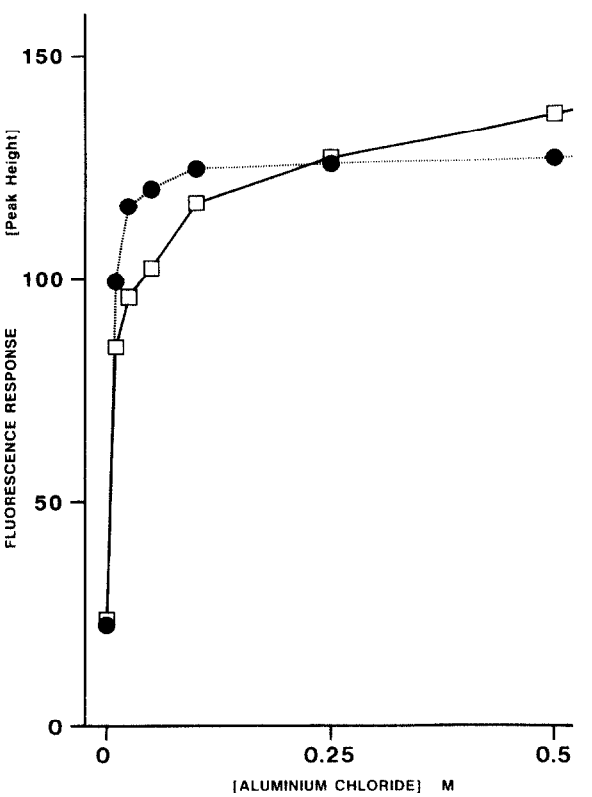


Fig. 3. Effect of aluminium chloride concentration on the post column derivatization of (□) zearalenone and (●) zearalenol

EXPERIMENTAL

Apparatus

High-performance liquid chromatography. The instrumentation consisted of pumps, system controller and U6K injector (Waters Assoc., Harrow, UK), a Shimadzu RF 530 fluorescence detector (Dyson Instruments, Houghton-le-Spring, UK) and a stainless-steel column packed with Spherisorb 5-μm ODS-1 (250 mm × 4.6 mm I.D.) (Hi-Chrom, Reading, UK), or similar HPLC equipment.

Post-column reaction system [13]. This was composed of an SSI stainless-steel tee-piece (1/16 in. × 0.0015 in.) (Jones Chromatography, Hengoed, UK), a PTFE coil (5000 mm × 0.3 mm I.D.) heated in a water-bath and an HPLC pump.

Materials

Solvents. Methanol and water of HPLC grade obtained from Rathburn Chemicals (Walkerburn, UK) or equivalent materials were used.

Aluminium chloride solution for post-column reaction. Aluminium chloride hexahydrate, 99.999% (Gold Label, Aldrich, Gillingham, UK) dissolved in methanol–water (3:1) to make a 0.25 *M* solution was used.

Mycotoxin standards. Zearalenone and zearalenol were obtained from Sigma (Poole, UK) and working standard solutions of 0.1 and 1.0 μg/ml zearalenone and 1.0 and 10.0 μg/ml zearalenol in methanol–water (80:20) were prepared.

High-performance liquid chromatography

HPLC was carried out using a Spherisorb 5-μm ODS-1 column with methanol–water (80:20) as

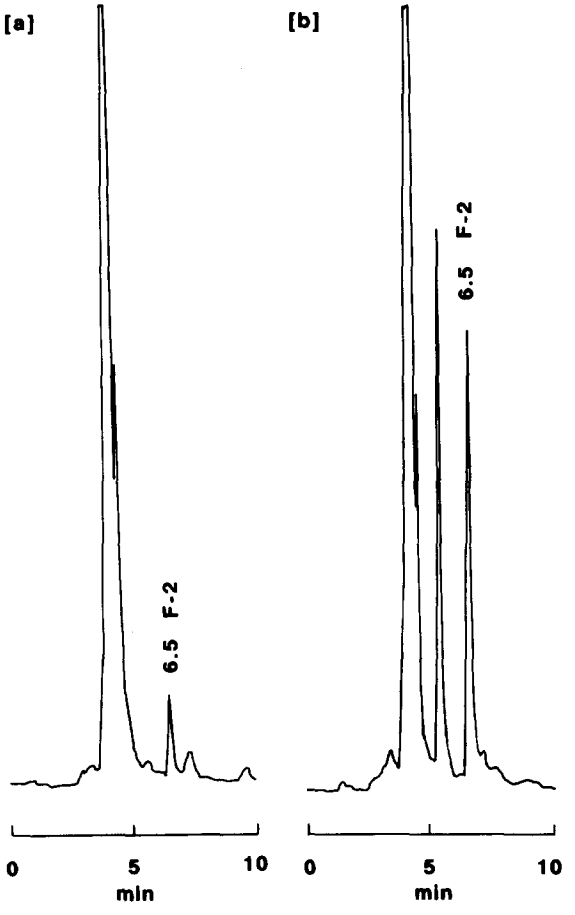


Fig. 4. HPLC of a mycotoxin-free maize extract with zearalenone standard equivalent to 100 μg/kg added, (a) without derivatization and (b) after post-column derivatization with 0.25 *M* aluminium chloride solution.

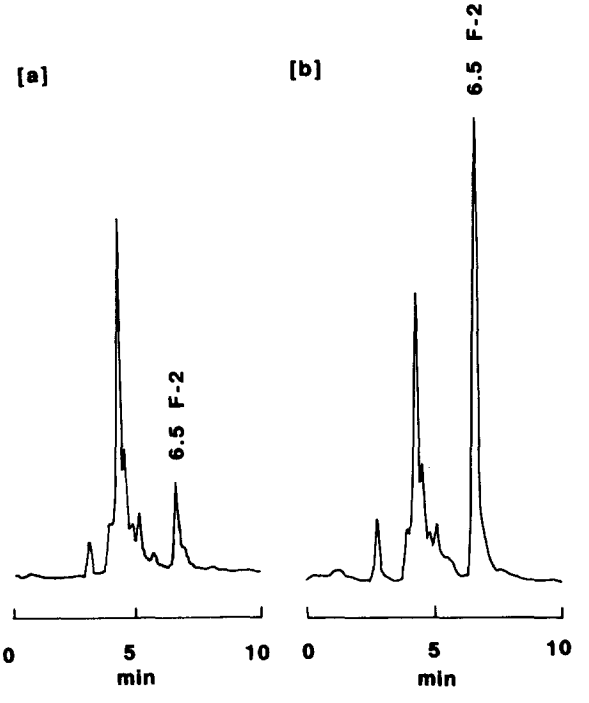


Fig. 5. HPLC of a mycotoxin-free wheat extract with zearalenone standard equivalent to 100 μg/kg added, (a) without derivatization and (b) after post-column derivatization with 0.25 *M* aluminium chloride solution.

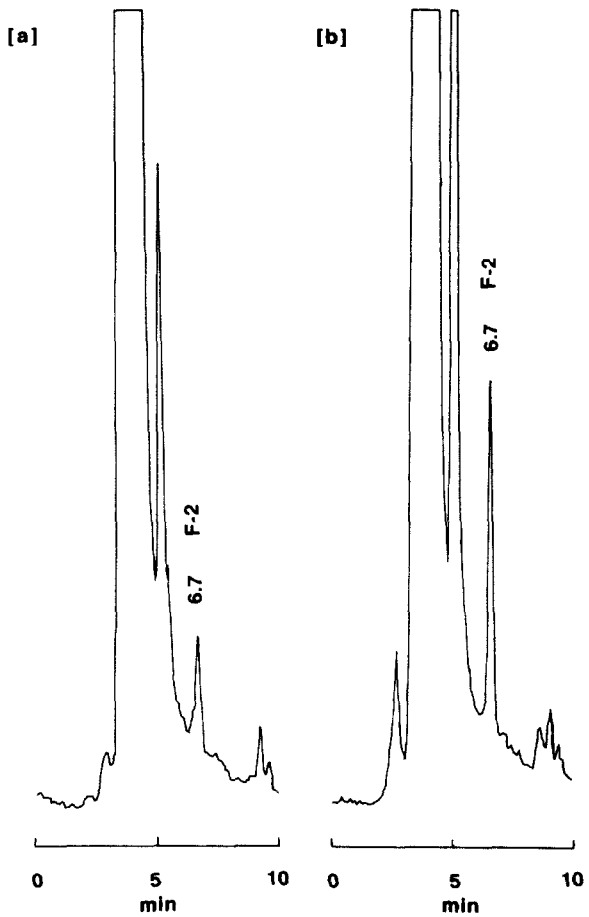


Fig. 6. HPLC of a compound feed extract naturally contaminated with zearalenone at a level of 50 μg/kg, (a) without derivatization and (b) after post-column derivatization with aluminium chloride solution.

eluent at a flow-rate of 1.0 ml/min. Detection of zearalenone and zearalenol was by fluorescence after post-column derivatization. A solution of aluminium chloride hexahydrate was pumped at 0.5 ml/min into the column eluent via a tee-piece as used previously for aflatoxins [13]. The combined column eluate and aluminium chloride solution was passed through a heated PTFE reaction coil held at 50°C in a water-bath and into the cell of the fluorescence detector, with the excitation wavelength set at 285 nm and emission wavelength at 440 nm. The system was flushed out by pumping methanol–water (3:1) to displace the aluminium chloride hexahydrate solution at the end of each working day as a precaution against corrosion and precipitation of aluminium chloride in the HPLC equipment.

Extraction and clean-up of commodity extracts

Extracts for examination were prepared either using a method published by Patterson and Roberts [5] for the multiple screening of a range of mycotoxins or gel permeation chromatography as described previously for aflatoxins [14].

RESULTS AND DISCUSSION

The increase in response for zearalenone and zearalenol obtained by post-column derivatization with aluminium chloride solution was found to be dependent both on the temperature of the reaction coil and on the concentration of aluminium chloride solution used. Fig. 2 shows the post-column fluorescence for zearalenone and zearalenol (25 and 250 ng, respectively) using 0.25 *M* aluminium chloride solution with reaction coil temperatures from 25 to 70°C. The optimum temperature for this reaction occurs between 50 and 60°C for zearalenone, and is slightly lower for zearalenol.

The effect of the concentration of aluminium chloride is shown in Fig. 3. A rapid increase in sensitivity occurred up to *ca.* 0.1 *M* concentration, after which the fluorescence increased linearly but at a slower rate. The maximum response for zearalenone (25 ng) was not reached. However, 0.25 *M* was selected as the working concentration, as a large increase in aluminium chloride concentration would be required for a relatively small further increase in fluorescence. The effect of concentration on the post-column response for zearalenol (250 ng) was similar, although the optimum response was apparently reached at a lower concentration.

Extracts prepared from non-mouldy, mycotoxin-free samples of maize and wheat using gel permeation chromatography [14] were examined with and without the post-column reaction. Figs. 4 and 5 show the chromatograms from these extracts of non-mouldy maize and wheat, respectively, to which zearalenone standard representing a level equivalent to 100 μg/kg in the original sample had been added. The selectivity of the post-column reaction, (a) without derivatization and (b) using 0.25 *M* alumi-

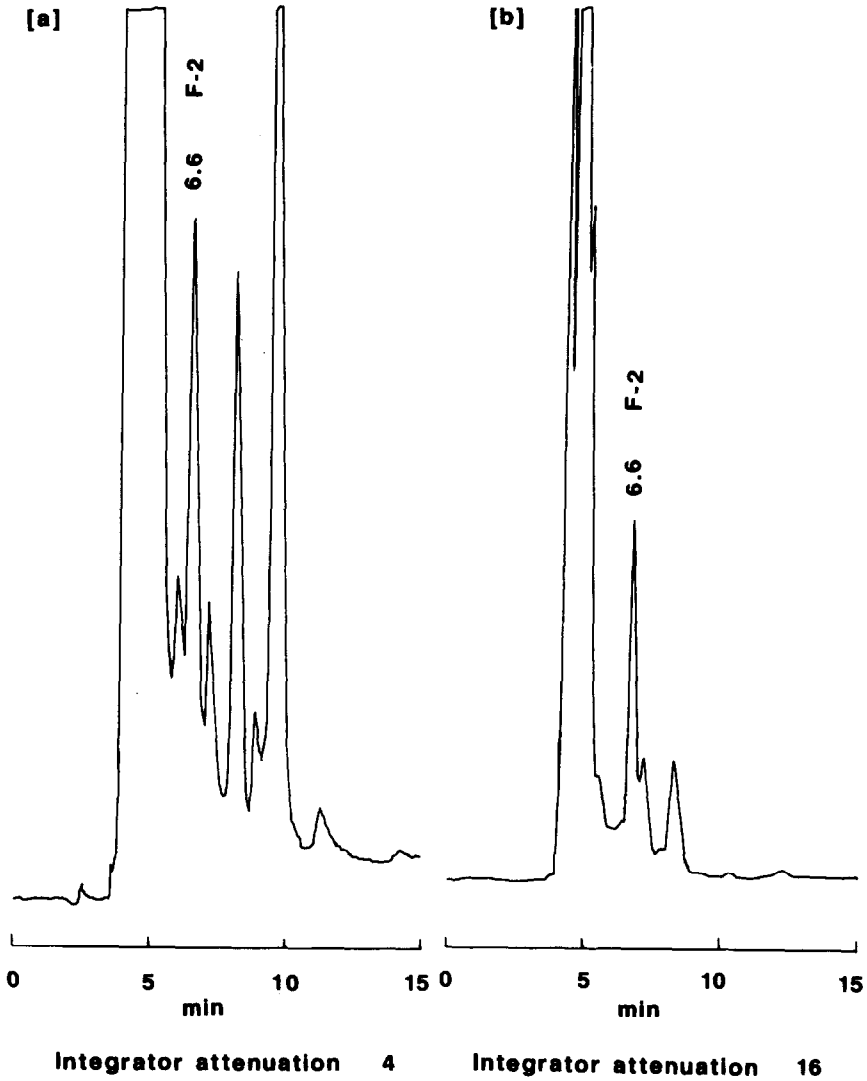


Fig. 7. HPLC of maize gluten extract naturally contaminated with zearalenone at a level of 150 μg/kg, (a) without derivatization and (b) after post-column derivatization with aluminium chloride solution.

nium chloride, is illustrated. Although the peak for zearalenone has increased *ca.* fivefold, the level of background interference from co-extractives is almost unaltered. However, with maize (Fig. 4) and also in the compound feed sample (Fig. 6), the response for a compound eluting about 1 min before zearalenone and close to the retention time of zearalenol is considerably enhanced by derivatization. The identity of this compound is unknown. However, it would potentially interfere with the determination of zearalenol.

A number of samples of cereals and animal feedstuffs were examined for zearalenone after clean-up either by gel permeation chromatography or using the method of Patterson and Roberts [5]. The chromatograms obtained for extracts of an animal feed sample prepared by the latter procedure are shown in Fig. 6. The sample contained *ca.* 50 μg/kg

of zearalenone, and the increase in response for zearalenone relative to the background interference from co-extractives permits a low limit of detection to be achieved and facilitates quantification at this level.

A sample of maize gluten naturally contaminated with zearalenone at a level of *ca.* 150 μg/g was extracted and prepared using gel permeation chromatography [14] and the chromatograms obtained without derivatization and after reaction with aluminium chloride are shown in Fig. 7. The volume of extract injected was the same in each instance but the sensitivity of the detector output was reduced to enable the peak of zearalenone after derivatization (Fig. 7b) to be compared directly. The peak for zearalenone after derivatization was considerably smaller than expected, and it is almost certain that another peak co-eluted with zearalenone, giving an artificially large response when monitored without derivatization as shown in Fig. 7a. After derivatization there is reduced interference at this retention time, and a more accurate response for zearalenone is obtained. The interference from other co-extractives is much reduced relative to the response for zearalenone.

Attempts to elucidate the nature of the chemical reaction involved in derivatization have proved unsuccessful to date. Collection of the zearalenone and zearalenol peaks eluting from the detector after post-column derivatization followed by extraction with chloroform, concentration and TLC resulted in a mixture of spots, including some corresponding to unreacted zearalenone and zearalenol. Aluminium chloride is a powerful Lewis acid and it may form a conjugate with zearalenone and zearalenol under the HPLC derivatization conditions. We have found a similar increase in sensitivity after derivatization with aluminium chloride for the mycotoxin sterigmatocystin.

CONCLUSIONS

The post-column reaction of zearalenone and zearalenol with aluminium chloride under the conditions described leads to an increase in fluorescence response of up to fivefold for these mycotoxins, without significantly affecting the level of background interference from co-extractives from cereal and animal feed samples. The post-column derivatization technique should be applicable to the determination of zearalenone and zearalenol in sample extracts prepared by a range of alternative analytical procedures and represents a significant improvement in limits of detection compared with most currently used procedures.

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