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NONDESTRUCTIVE DISTINCTION BETWEEN AFLATOXIN B_1 AND ETHOXYQUIN IN THIN-LAYER CHROMATOGRAPHY

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SUMMARY

A rapid and simple method has been developed for the nondestructive distinction between aflatoxin B_1 and the feed antioxidant, ethoxyquin. These two chemicals exhibit similar R_F values in certain solvent systems and produce a similar bluish fluorescence under long UV (366 nm) radiation. The method involves the *in situ* generation of fluorescence spectra of the respective thin-layer chromatography spots. Since it is nondestructive, the method affords ancillary study of the separated aflatoxins.

INTRODUCTION

Aflatoxins, known toxic metabolites, are produced by a small number of fungi, notably *Aspergillus flavus*. Extensive studies were initiated in 1960 when these materials were implicated as the cause of "Turkey X Disease", which resulted in the death of an estimated 0.1 million turkeys in Great Britain[1]. Since that time, research has demonstrated toxic[2] and carcinogenic[2] properties of aflatoxins. Aflatoxin B_1, the most toxic of all the aflatoxins[2], biochemically binds to DNA[3], inhibits DNA[4], RNA[5], and protein synthesis[5], and affects DNA polymerase activity[6].

The presence of feed additives (*e.g.*, ethoxyquin, a feed antioxidant) can result in analytical errors in the qualitative and quantitative analysis of afltoxin residues in animal feeds and peanut meals. Shotwell *et al.*[7] observed two unidentified components in oats which produced similar analytical problems. Our present study shows that ethoxyquin [1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline] produced the same bluish fluorescence as aflatoxin B_1, and exhibits essentially the same R_F value in several solvent systems. An R_F value of nonaflatoxin component, similar to that of aflatoxin B_1, can lead to erroneous analytical conclusions regarding the presence of aflatoxin B_1 and its actual amount in the sample. Over the past few years, several chemical methods for the identification of aflatoxins B_1, B_2, G_1 and G_2 have been reported. However, most of these methods are time-consuming, involve undergoing a second development or spraying with one or more reagents, and are destructive. A more rapid,

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simpler, *in situ*, and nondestructive method for the qualitative and quantitative analysis of aflatoxin residues, is the subject of this report.

EXPERIMENTAL

Apparatus

A Perkin-Elmer Model MPF-3 fluorescence spectrophotometer having a thin-layer chromatography (TLC) scanning attachment and strip chart recorder was used as the analytical system. The optimum instrumental conditions observed were as follows: excitation slit, 6 nm; excitation wavelength, 366 nm; emission slit, 6 nm; emission wavelength, 420 nm; plate scanning speed, 25 mm/min; wavelength scan, 30 nm/min. A viewing cabinet with long-wavelength (366 nm) and short-wavelength (254 nm) UV lamps (Brinkmann, Westbury, N.Y., U.S.A.) was also used.

Reagents

All solvents used were glass-distilled (Burdick and Jackson, Muskegon, Mich., U.S.A.). All reagents were analytical grade. Aflatoxin TLC standard kits (Supelco, Bellefonte, Pa., U.S.A.) for B_1 and G_1 (1 μg/ml), and B_2 and G_2 (0.3 μg/ml) were used. Stock solutions (Pfaltz and Bauer, New York, N.Y., U.S.A.) of ethoxyquin (5 μg/ml) were prepared in chloroform–acetonitrile (98:2). Drummond Gold Label micropipettes were used for spotting the samples on silica gel plates (Sil-G-25-HR; Brinkmann). The TLC plates were used without pretreatment.

Procedure

Aflatoxin standard and ethoxyquin standard solutions (5 μl each) were spotted under subdued light, and developed in a nonsaturated equilibrated tank in *ca.* 100 ml of the appropriate solvent mixture. The plate was removed after development and allowed to air-dry in the dark, after which it was scanned and the fluorescence spectra of the resolved components were recorded. It was observed that nonactivated plates gave a better separation of the aflatoxins than heat-activated plates.

RESULTS AND DISCUSSION

The TLC results from several solvent systems yielding close R_F values for aflatoxin B_1 and ethoxyquin are given in Table I. It is apparent from such results that the chromatographic separation of these two chemicals may be difficult in solvent systems useful for the separation of aflatoxins B_1, B_2, G_1, and G_2. The best solvent system found (chloroform–acetone–water, 88:12:1.5) produces separation of these aflatoxins, as shown in Fig. 1.

There are several methods to identify aflatoxins B_1, B_2, G_1, and G_2. These may be divided into four categories: (a) visual, (b) instrumental (fluorodensitometric scanning), (c) derivative formation, and (d) solvent mixtures (R_F). The first of these (visual methods) is based on the strong natural fluorescence exhibited by aflatoxins under long-wavelength UV light. Aflatoxins B_1 and B_2 were so named since they produce a blue fluorescence, while aflatoxins G_1 and G_2 produce a green fluorescence. The aflatoxin subscripts 1 and 2 refer to their respective R_F order when developed on TLC plates in most solvent systems. Of the four primary aflatoxins, aflatoxin B_1 has

TABLE I

R_F DATA OF AFLATOXIN B_1 AND ETHOXYQUIN

Solvent system	*Aflatoxin B_1*	*Ethoxyquin*
Chloroform–acetone–hexane (85:15:20)	0.53	0.58
Chloroform–acetone–propan-2-ol (85:15:20)	0.53	0.58
Chloroform–acetone–water (88:12:1.5)	0.66	0.68

the highest R_F value and aflatoxin G_2 the lowest. Although visual detection is the easiest and fastest of the above four methods, it can be subject to error as a result of fluorescing impurities and operator variances. In quantitative measurements, a possible error of 30–50% can occur when an unknown is visually compared to one of two standards[8] of different concentrations, and a possible error of 15–25% can occur when an interpolation is made between two such standards[9].

Fluorodensitometric scanning is far more accurate and precise than the visual method. The two general modes of this technique, transmission and reflectance, have both been shown to yield similar results[10]. Each mode is capable of steadily scanning the TLC plate such that the recorder output yields a scan of fluorescence intensity as the spot traverses the light beam. The precision in measuring individual known amounts of aflatoxins as spots on TLC plates has been reported as ± 4–10%[11,12]. The advantages of this method are: (a) linearity over the range of concentrations usually encountered in TLC analysis (*ca.* 0.1–20 ng/spot for aflatoxins B_1 and G_1 and 0.3–7.5 ng/spot for aflatoxins B_2 and G_2)[10,13] and (b) reproducibility (± 4–5% for aflatoxins B_1 and G_1 and 5–9% for aflatoxins B_2 and G_2)[10,13]. Since aflatoxins are sensitive to UV irradiation, some degradation (*ca.* 1% per scan[10]) can occur. This is particularly true for aflatoxins B_1 and G_1, but not for aflatoxins B_2 and G_2. It was observed after scanning these four aflatoxins three times, followed by plate development in chloroform–acetone–water (88:12:1.5), that aflatoxins B_1 and G_1 each gave an additional significant spot, while aflatoxins B_2 and G_2 each gave a single spot having the

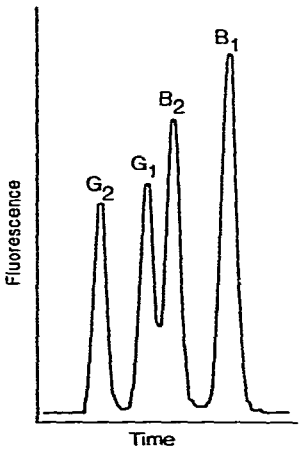

Fig. 1. Fluorometric scan of the separation of aflatoxins on silica gel using the solvent system chloroform–acetone–water (88:12:1.5).

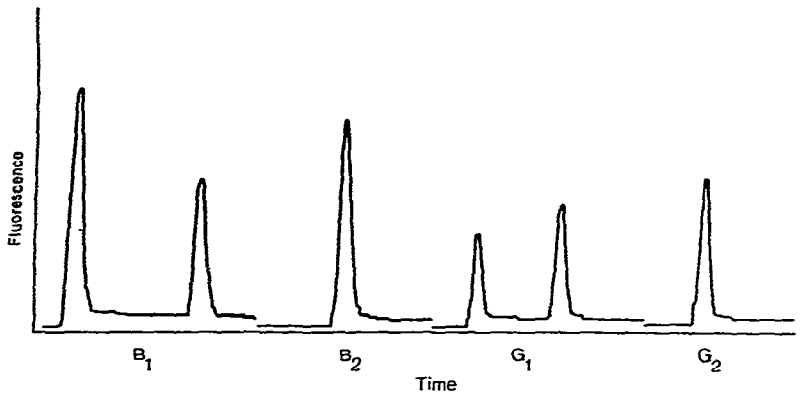

Fig. 2. Fluorodensitometric scanning (reflectance mode) of aflatoxins B_1, B_2, G_1 and G_2 after three prior scans for each aflatoxin, followed by plate development with chloroform–acetone–water (88:12:1.5).

R_F value of the authentic aflatoxin (Fig. 2). Even if the amount of degradation is small, this should be taken into account if plates are to be stored and rescanned. Developed plates should be scanned as soon as possible following development if quantitative results are required. However, if they have to be stored prior to analysis, they should be placed under subdued light and covered with clear glass plate[14].

The third identification method is aflatoxin derivative formation. There is a large number of potential derivatives, a few of which will be discussed here. Some derivatives are formed by reaction with the vinyl ether double bond of the furan ring. Some of the early derivatization reactions utilized were formic acid–thionyl chloride[15,15], acetic acid–thionyl chloride[15,16] or trifluoroacetic acid[15,16]. The reaction of aflatoxin B_1 with acetic acid–thionyl chloride yields two fluorescent products having two R_F values between aflatoxins B_1 and G_1. Reaction of aflatoxin B_1 with formic acid–thionyl chloride or with trifluoroacetic acid yields a single fluorescent spot approximately one tenth the R_F value of aflatoxin B_1[16]. These reactions are also known to occur with aflatoxin G_1; however, reactions with thionyl chloride do not usually go to completion, whereas the trifluoroacetic acid reaction does[17].

Spray reagents which react at the double bond of the furan ring have also been studied for developed plates. For example, 25% aqueous H_2SO_4 changes the aflatoxin B_1 and G_1 fluorescence color from blue and green (respectively) to yellow[18]. The acid-catalyzed addition of water to this double bond to yield aflatoxins B_{2a} and G_{2a} has also been studied[19] and is considered as a confirmatory test[15]. Acid-catalyzed methanol and ethanol derivatives have also been prepared[19]. It was also shown that O-alkyl derivatives can be formed by reaction with small amounts of alcohol present in chloroform solvent[19]. Care should be taken with regard to impure solvents since small losses of aflatoxins B_1 and G_1 could presumably occur during solvent extraction of aflatoxin residues from natural products. Oxime and 2,4-dinitrophenylhydrazone derivatives[20], which involve reactions at the carbonyl group on the cyclopentanone ring[21], have also been studied. These reactions will occur with aflatoxins B_1 and B_2, but not with the lactone carbonyl in aflatoxins G_1 and G_2.

The fourth method requires the use of more than one solvent system for identification. Comparison of the data between two or more solvent systems can help to

distinguish between those spots produced by aflatoxins and those produced by non-aflatoxin impurities. As previously shown in Table I, aflatoxin B_1 and ethoxyquin have close R_F values in several solvent systems. On the other hand, chloroform–hexane–acetone–water (80:30:10:2)[22] gives R_F values for aflatoxin B_1 and ethoxyquin of 0.27 and 0.53, respectively. Furthermore, a solvent system of benzene–methanol–acetic acid (90:5:5)[23] on silica gel was also shown to resolve aflatoxin B_1 (R_F 0.27) and ethoxyquin (R_F 0.01). It is notable that the latter two solvent systems are inferior for the separation of aflatoxins B_1, B_2, G_1 and G_2 to those used in Table I.

While the four identification categories describe methods to distinguish between aflatoxin B_1 and ethoxyquin, they require the use of derivatives, spray reagents or a second development. It is felt that a better approach is offered by fluorescence spectroscopy, used in the following manner. Ethoxyquin fluoresces with the same bluish color as aflatoxin B_1 and their emission maxima are similar (420 nm), but their respective fluorescence spectra produced by scanning the excitation wavelength range of 300–400 nm at a fixed emission wavelength (420 nm) are different.

Aflatoxin B_1 was found to have a fluorescence excitation maximum at 366 nm, which agrees with the wavelength reported earlier[24], while that of ethoxyquin was at 348 nm (Fig. 3). Scanning is completed in less than 5 min; fluorescent spots can be tested during a scan without removal of the plate from the densitometer. This method represents a rapid and simple way to distinguish aflatoxin B_1 from ethoxyquin in a nondestructive manner. When the resultant spectrum appears dissimilar to either scan shown in Fig. 3, another aliquot of the sample is rechromatographed in benzene–methanol–acetic acid (90:5:5), which readily resolves aflatoxin B_1 and ethoxyquin. The fluorescence spectrum of the resultant spot(s) is then obtained for confirmation of spot identity. This method of validating aflatoxin B_1 is simple, conclusive, and enhances the validity of levels reported in residue analysis of foods and feeds for this important carcinogen.

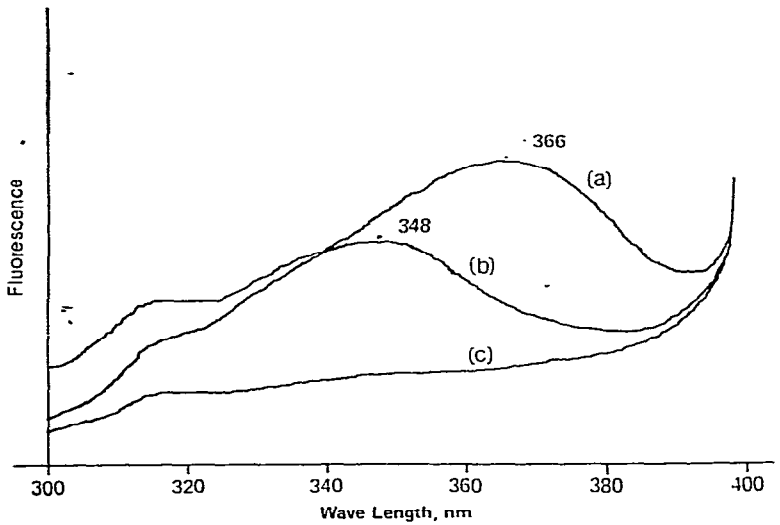

Fig. 3. Fluorescence excitation spectra of aflatoxin B_1 (a), ethoxyquin (b), and background (c) at a fixed emission wavelength of 420 nm.

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