STUDIES ON AFLATOXIN, STERIGMATOCYSTIN AND AFLATOXIN-PRODUCING FUNGI IN ANIMAL FEEDSTUFFS IN JORDAN

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By

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STUDIES ON THE AFLATOXIN, STERIGMATOCYSTIN AND AFLATOXIN-PRODUCING FUNGI IN ANIMAL FEEDSTUFFS IN JORDAN

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ABSTRACT

Six hundred and six feed samples of ten different types of animal feedstuffs collected from different areas in Jordan were tested for aflatoxins, sterigmatocystin and aflatoxin-producing fungi. One corn sample was found to be contaminated with aflatoxin B_1 (AFB_1) and aflatoxin B_2 (AFB_2) with concentration of about 53 and 13 ppb, respectively. Sterigmatocystin was not detected in all samples tested. Probable aflatoxin producing fungi were sought in these samples and on the basis of microscopic and morphological identification, 238 isolates of the *Aspergillus flavus* group were obtained. Representative isolates were identified and confirmed by the Commonwealth Mycological Institute, Kew, England. These appeared to belong to *Aspergillus flavus* link ex fries. Some were identified as *Aspergillus parasiticus* spear, *Aspergillus oryzae* or *Aspergillus tamarii*. Twenty two (9.2%) of the isolates were found to be aflatoxin producers. The suitability of some feedstuffs as substrate for aflatoxin production was also studied, these included poultry concentrate, poultry pellets, wheat bran, and lentil. All were good substrate for aflatoxin production.

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Toxins produced naturally on the feed, natural substrates in the laboratory and synthetic laboratory media were identified by thin layer chromatography (TLC), chemical confirmation tests and UV-absorption. The majority of the aflatoxigenic isolates were capable of producing AFB_1 and/or AFB_2. Some isolates produced AFB_1, AFB_2 and AFG_1.

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INTRODUCTION

Aflatoxins can be defined as a group of metabolites produced by certain species of <u>Aspergillus</u>. Sterigmatocystin also is a metabolite produced by few fungal species but mainly by <u>Aspergillus versicolor</u>. The genus <u>Aspergillus</u> that includes these toxigenic species has been studied thoroughly and found to contain one hundred and thirty two species. (Raper and Fennell, 1965). Both types of toxins produced by different species; have been the subject of investigation due to the widespread contamination of human foodstuffs and animal feeds (Hsieh <u>et al</u>., 1973).

The ubiquitous nature of the molds which infest a wide range of animal feeds and human foods resulted in the speculation about the involvement of aflatoxins and sterigmatocystin in the etiology of liver disease (FDA, 1978). The potent hepatoxic and carcinogenic activity of aflatoxin B_1 confirms this belief. Sterigmatocystin also found to be carcinogenic to rat (Purchase and Van der Watt, 1973; Dickens <u>et al</u>., 1966) and also to vervet monkey; (Van der Watt and Purchase, 1968 and 1970). Histologically the tumors bore a marked resemblance to those occurring in man (Van der Watt, 1970a). Sterigmatocystin was produced by large quantities on

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different substrates including different vegetables, fruits, maize,and fruit juice (Thurn *et al.*, 1979). Grapes and fruits in general were classified as good substrate for sterigmatocystin production by *A. versicolor* (Paul *et al.*, 1979).

Aflatoxins occur in a variety of crops, meat, milk, and eggs of animals fed on aflatoxin containing feed. They also occur in cereals, nuts as a major substrate for aflatoxin production. The toxic effect of both toxins and their occurrence in feeds and foods together with their involvement in hepatoma and other diseases justify studies which help revealing the degree of contaimination of such toxins. In Jordan no work of this nature has been done as a result it would be a very useful study to clarify any problem, if there is any, concerning the contamination of feedstuffs with these toxins and the presence of fungi capable of producing such toxins in these feeds. In this study an attempt is presented to cover the following :

1. To have an idea about the major types of animal feeds used in Jordan.
2. To determine the incidence and amounts of aflatoxins and sterigmatocystin in these feedstuffs used commonly.

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3. The occurrence of aflatoxin producing fungi in some feedstuffs and their suitability for aflatoxin production.
4. Finally to evaluate the situation concerning the use of these animal feedstuffs and to be aware of any problems involved.

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LITERATURE REVIEW

Systemic study of the fungi during the last twenty years, or so, has resulted in the discovery of many new varieties and species (Raper and Fennell, 1965). The genus Aspergillus consists of eighteen varieties and one hundred and thirty two species (Heatheote and Hibbert, 1978). Several of these species such as A. flavus, A. niger, and A. fumigatus are animal and human pathogens which are responsible for a number of diseases collectively known as Aspergillosis (Raper and Fennell, 1965). A large number of fungi, including the genus Aspergillus, are capable of producing a large number of primary and secondary metabolites (Heathcote and Hibbert, 1978). Interest in the isolation and characterization of secondary metabolites of micro-organisms has increased rapidly over the last few years, the reason being that the fungal contamination of feed and food has been found to cause mycotoxicosis in both animal and man (Goldblatt, 1969). Prior to 1960 very little was known about the structure of the toxic metabolites, until some alarming reports came from farms in the south east of England. More than 100,000 Turkeys in these farms died in the course of a few months from an apparently new disease that was primarily named : Turkey "X" disease, because this disease was not reported before (Blount, 1961).

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Similar reports appeared at the same time from Kenya and Uganda indicating severe loss of ducklings from a similar disease (Blount, 1961). Deaths on such scale, together with their serious economic implications, stimulated intensive investigation by scientists in many countries. Eventually the toxic metabolites responsible for the disease were isolated and called "AFLATOXINS" as they are Aspergillus flavus toxins (Heathcote and Hibbert, 1978). As a result of the continuing analysis about such metabolite of A. versicolor was characterized by Hatsuda and Keyama, 1962 and was found to be a derivative of difuranoxanthone. Sterigmatocystin is structurally related to aflatoxins, as it is clear in Figure No. 1 (Bulock, 1962). In 1972 Scott et al., reported for the first time the presence of sterigmatocystin (St) in wheat and showed that this toxin has great similarity to already known toxins. Extracts from toxin-containing feed could be split into four main components. Hartley et al., (1962) chromatographed these extracts on thin layer silica gel plates and developed them in a chloroform methanol mixture. Two of these components with Rf values of 0.4 and 0.36 fluoresced blue under UV-light and were designated AFB_1 and AFB_2, respectively, the two with slightly lower Rf values of 0.34 and 0.31 fluoresced turquoise-green under UV-light and were

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designated AFG_1 and AFG_2, respectively.

Some species of the genus Aspergillus produce aflatoxins in the laboratory, but A. flavus and A. parasiticus are the only aflatoxin producing fungi known to be associated with aflatoxin production in nature (Kulik and Holaday, 1967). Sterigmatocystin has been isolated from A. versicolor, A. rugulosus and Bipolaris sorokiniana (Scott et al., 1972, Ballantine et al., 1965). The most important group in aflatoxin production is the A. flavus group. They are a part of the soil and air microflora and occur in living and dead plants and animals and are capable of surviving under variable climatic conditions (Raper and Fennell, 1965). The fungi from which the sterigmatocystin has been isolated are ubiquitous in nature (Raper and Fennell, 1965) and high concentration of the toxins is produced on natural and synthetic substrates (Theil and Steyn, 1973). A. parasiticus produces four types of aflatoxins B_1, B_2, G_1 and G_2 and most isolates were reported to be toxins producers. While A. flavus produce mainly B_1 and B_2 with a greater possibility to have non-producer strains if compared with A. parasiticus (Goldblatt, 1969). The mere presence of moldiness is not, by itself; indicative of toxin production. The natural occurrence of mycotoxins can be described as such only when the initial inoculum by the

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mold spores and subsequent mold development and toxin production are a natural sequence of events (Heathcote and Hibbert, 1978). The natural occurrence of mycotoxins on natural substrates is controlled by many factors. One factor is the contaimated strain. A. parasiticus NRRL 2999 produces the four major types of aflatoxins, while A. flavus link ex fries produces B_1 and/or B_2 (Goldblatt, 1969). A. tamari and A. oryzae, although producing some aflatoxins on synthetic media, do not produce toxin on natural substrates (Goldblatt, 1969).

The substrate is another factor in toxin production Hesseltine et al., (1966) showed that the same mould, A. flavus NRRL 2999; produced different amounts of toxins on different substrates. It produced more aflatoxin on peanut, rice, sorghum and soybean than on wheat and corn, while the strain A. flavus 3000 produced more aflatoxin on wheat and corn. This proved that there is a kind of "interaction" between the strain and the substrate, (Goldblatt, 1969).

Moisture is also a crucial factor for the growth and toxin production of all fungiof the A. flavus group. The optimal moisture content of substrate for aflatoxin production is 18% and the minimal moisture content is 8%

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(Ciegler, 1977). The optimal relative humidity for fungal growth is 85% or greater and the lower relative humidity for aflatoxin production on peanuts is 83% (Diner & Davis, 1968).

Aeration influences aflatoxin production directly (Ciegler *et al.*, 1966). Cultivation of *A. flavus* on a medium of groundnuts in an atmosphere depleted of O_2 showed that the production of aflatoxin was greatly reduced when compared with controls grown in normal atmosphere. Significant decrease resulted when the O_2 was reduced from 5 to 1% (Heathoote, & Hibbert, 1978). Increasing the carbon dioxide concentration from 0.03 (normal atmosphere) to 20% resulted in decreasing the production of aflatoxin (Heathcote & Hibbert, 1978).

The optimal temperature for aflatoxin production is mold species dependent. *A. flavus* produced maximal amounts of toxins in culture flasks at 25^{0}C for 7-9 days. Increasing the temperature to 30^{0}C resulted in decreasing the time to 5-7 days (Diener and Davis, 1966). *A. parasiticus* produced maximum AFB_1 at 30-35^{0}C and maximum AFG_1 at 25-30^{0}C, however large amounts of total aflatoxin were produced at 25-30^{0}C during incubation period of 7-15 days (Heathcote & Hibbert, 1978). The lowest temperature for aflatoxin production on

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peanuts by *A. flavus* was reported to be 13 ± 1°C and the upper limiting temperature was 41.5 ± 1.5°C (Diner & Davis, 1967). Competition between fungi for the substrate under favorable environmental conditions will restrict the amount of aflatoxins formed (Goldblatt, 1969). The presence of aflatoxin B_1 on the substrate inhibited the growth of many fungal species including *A. flavus* and *Penicillium* spp. (Lillehoj *et al.*, 1967a).

Animals that are consuming AFB_1 in their feed secreat a toxin in their milk called "Milk toxin M_1". It was also isolated from their urine and their tissues (de Iong *et al.*, 1966). This M_1 toxin produced symptoms in ducklings indistinguishable from those produced by known aflatoxins (de Iong et al., 1966). Also when lactating rats are fed on feed contaminated by AFB_1 they transfer it to AFM_1 and later it was isolated from their livers (Butler and Clifford, 1965). This toxin causes typical bile duct proliferation. Tissue culture made from human liver cell was found to transfer this AFB_1 to AFM_1 (Merril and Campell, 1974). The AFM_1 toxin that is secreted with milk and urine causes renal necrosis in ducklings. It also causes hepatocarcinoma in trouts and sarcoma in rats (Bodine et al., 1977).

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Studies have shown that tissues from sheep, swine, and chicken which ingest aflatoxin-contaminated feed may contain low levels of AFB_1, AFB_2, AFG_1, and AFG_2 or their metabolized: AFM_1 or AFM_2 forms (Brown <u>et al</u>., 1973). In a study Brown <u>et al</u>., (1973) found that 24 sheep which were fed on aflatoxin-containing cotton seed; AFB_1 was present in all kidney and liver samples. AFM_1 was extracted from heart, liver, and kidney. The secretion of AFM_1 in the milk increases the danger to the sucking newborne lambs and to the consumers of these sheep and their milk.

Sterigmatocystin was proved to be carcinogenic to rats when administered by repeated subcutaneous injection (Dickens <u>et al</u>., 1966) but the activity was 1/250 of that of AFB_1 administered through the same route. Oral administration of the sterigmatocystin was also hepatocarcinogenic to rat (Purchase & van der Watt, 1973) with a potency greater than 1/10 of that of AFB_1 given orally. Tumors other than hepatocellular carcinoma were also seen in various organs, and generally speaking, in experimental animals, males were found to be more susceptible than females (Purchase and van der Watt, 1973). Histologically, the tumors have marked resemblance to those occurring in man in Africa (Purchase and van der Watt, 1973). Sterigmatocystin in vevert monkey produced primarily hepatic necrosis, also it was found to be carcinogenic to rainbow trouts (Hendrick, 1980). Papillomas and squamous cell carcinoma were observed when sterigmatocystin was given to these animals subcutaneously (Hendrick, 1980).

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A comparative study of the cytotoxic effect of sterigmatocystin and AFB_1 on nucleolar morphology, mitosis and DNA synthesis of primary cell culture showed that both caused nucleolar changes and inhibition of mitosis. Sterigmatocystin was more effective than aflatoxin B_1 in this regard (Engelbrecht and Altenkirk, 1972). The incidence and levels of aflatoxin contaminations very markedly from one region to another. It seems that aflatoxin contamination is a problem for tropical and semi-tropical areas (Goldblatt, 1969) as would be expected from the temperature preference of the producing moulds. The strong carcinogenic effect of aflatoxins to man and animals pushed many countries to adopt control measures to reduce toxin contamination. Many countries established laboratories near the sea-ports to examine the imported feed and food that could be a suitable substrate for toxin production (Danesh et al., 1979).

Several studies have been conducted on a number of feed and foodstuffs in many countries like U.S.A (Hesseltine, 1975) Canada (Funnell _et al._, 1979), Australia (Bryden _et al._, 1980), India (Goldblatt, 1969), Tunisia (Boutriff _et al._, 1977), Iraq (Al Adil _et al._, 1976) and Jordan (Jarrar, 1980).

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Corn is consumed in large scales as feedstuff for poultry, and cows. It is a diet where aflatoxins are likely to be found (Heathcote & Hibbert, 1978). A. flavus group was isolated from many tested samples. It was isolated from 1276 out of 1283 samples tested for this purpose (Hesseltine, 1975). The occurrence of toxin producers in the field before harvest has been recorded (Hesseltine et al., 1976 and Mahasneh et al., 1980). Infection rates of 0.02-0.09% among corn kernels before harvest was reported by Tuite, (1961) and also other studies showed similar results (Tuite and Caldwell, 1971; Romb et al., 1974). Two hundred ninety seven corn samples were collected at harvest from seven counties in north-eastern South Carolina and dried to less than 13% moisture content as quickly as possible and tested for aflatoxin. One hundred fifty two were positive for aflatoxins, one hundred twenty of them showed one or more kernels internally infected with A. flavus, and 59 samples of the 145 negative samples showed infection by A. flavus. This study showed also that in 247 out of 375 insects, collected from corn; A. flavus was present, (Goldblatt, 1969). A preharvest sampling of corn at various locations in the U.S.A showed AFB_1 to exceed 20 ng/gm (20 ppb) at 3 of 13 (23%) locations in 1972, four of fifteen (27%) in 1973 and eleven of twenty one (52%) in 1974 (Zubber, 1976).

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It is now widely accepted that improper storage of feed leads to the invasion of the _A. flavus_, and if conditions are suitable, aflatoxins will be produced on corn. Until 1976, AFM_1 had not been reported to occur naturally in corn, in fact, the only commodities, other than milk in which AFM_1 had been detected, were peanuts and pistachionuts but in 1976 it was reported the occurrence of M_1 in 12 lots of corn from several sources (Heathcote & Hibbert, 1978).

Contamination of cotton-seed, a universal feedstuff, by AF is a world wide phenomenon. Contamination was found to be associated with poor agricultural practices and insect damage which results in ball rat caused by _A. flavus_ under high temperature and relative humidity. The incidence of contamination in affected areas can reach 80-100% of the total crop harvested (Stolloff, 1977). Soybeans appears not to be a good substrate for aflatoxin production and are also resistant to invasion by _A. flavus_. Oil seeds in general, support the production of aflatoxin less than the high carbohydrate substrates (rice, wheat). This probably because the oil seeds contain large percentage of oils that are not immediately metabolized by _A. flavus_ (Goldblatt, 1969). Aflatoxins were found to occur on soybean at low levels (7-14 ppb) only in two out of 1046 samples collected from different regions of U.S.A

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(Shotwell *et al*., 1978). Hesseltine *et al*., (1966) presented data showing that the amount of aflatoxin produced on soybean were 50-90% of the amount produced on corn, rice and wheat. Barley, and other small grains, like oat and wheat; do not seem to be a significant natural source of aflatoxin (Ciegler, 1977), still the toxin was detected at biologically significant levels in some samples of barly, sesame, sorghum and cowpeas (Goldbaltt, 1969).

Alfalfa failed to show the presence of aflatoxin (Hesseltine, 1968). Hesseltine (1968) pointed out the difficulties of utilizing alfalfa as substrate for the production of aflatoxins. Smally *et al*., (1972) suggested that the problem of aflatoxin analysis might not be caused by the alfalfa itself, but by the various fungal parasites or contaminants and their metabolites preserved on the hay when cut or during fermentation. A survey done by Smally *et al*., (1972) on alfalfa hay from different localities in Wisconsin, U.S.A; showed that 9% of 87 hay samples tested contained no culturally detectable *A. flavus* 56% contained a small, but detectable *A. flavus* group, 18% moderate *A. flavus* components and 16% contained *A. flavus* as a major micro-floral component. *A. fumigatus* was the fungus most frequently isolated from all samples in that survey. Two hundred thirty eight isolates of the *A. flavus* group

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were obtained from all the samples. Forty seven (20%) produced small to large amounts of AFB_1, few produced AFB_2 or AFG_1 but non produced AFG_2. Maximum AFB_1 production on inoculated hay occurred at 24-30% moisture and 16-24^{0}C, although mold development was greatest at 30-40% moisture content.

In a survey done by Bryden et al., (1980) in Australia, AFB_1 was detected in 23 out of 55 samples of different feedstuffs. The concentration reached up to 0.7 mg/kg but the mean was 0.14 mg/kg. Eleven feedstuffs were associated with field outbreaks of animal disease and the toxin was detected in seven of these animals. In Canada, Funnell et al., (1979) reported that 2022 samples of animal feedstuff were tested for mycotoxins during the period 1972-1977. Two hundred seventy seven samples of them (13.7%) were found to have one or more mycotoxin (aflatoxin, sterigmatocystin, ochratoxin...etc). The amount of aflatoxins was low and sterigmatocystin was absent. Another study done on animal feedstuff by Afzal et al., (1979) including rice, corn grain, corngluteen feed, cotton seeds, sesame oil cake, and wheat bran. The incidence of aflatoxin was in 60% in broken rice, 25% corn grain, 25% corn gluteen feed, and 23% in cotton-seed cake, but no aflatoxin was found in rice polish, sesame or wheat bran.

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Damaged samples revealed a much higher incidence (50%) as compared to undamaged ones (7.5%). Cultural examination of aflatoxin-positive feedstuff yielded 39 isolates of fungi. Twenty one of these were Aspargillus isolates, of which only one (4.7%) was toxin producer when tested on suitable synthetic medium and it was identified as A. parasiticus.

In Japan, A. flavus and A. versicolor with other fungi were found in almost all samples of mixed feed tested, these included barly, corn, soybean, alfalfa and fish meat (Takatori et al., 1980). Eight out of thirty one (25.8%) of A. flavus isolated were aflatoxin producers 11/19 (or 57.9%) of A. versicolor isolates were sterigmatocystin producers.(Takatori et al., 1980).

A. versicolor was isolated from herbal drugs (Horie et al., 1979) and produced, naturally; sterigmatocystin on nine samples out of 39 cheese samples at concentrations varying from five to 600 μg/kg (Northolt et al., 1980).

The growth of Aspergillus flavus group and aflatoxin production can be inhibited or decreased by using certain compounds such as insecticides like Dichlorovas at 100 ppm inhibited up to 90% the production on synthetic medium (Achmoody and Chipley, 1978); Citrus oil was found to

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inhibit to a certain degree fungal growth and aflatoxin production, also dimethyl sulfoxide inhibits AF production drastically but a high concentration was needed (Achmoody and Chipley, 1978). Sodium acetate was found to inhibit the production of aflatoxin by A. parasiticus 2999. P-amino-benzoic acid, potassium sulfite and potassium fluoride have the ability to inhibit aflatoxin production to a high degree (Achmoody and Chipley, 1978). Barium at the one-ppm level completely inhibited aflatoxin production with no effect on mycelial growth (Achmoody and Chipley, 1978). Natural inhibitors of Aspergillus flavus link ex fries from legumes have been characterized. For example, the presence of phytic acid in soybeans prevented heavy aflatoxin contamination of this important feedstuff. Smoking of natural and synthetic substrates resulted in significant reduction in toxin production by A. parasiticus NRR 2999 under a variety of cultural conditions (Achmoody and Chipley, 1978).

Metabolic inhibitors such as N, N-dicyclohexylcarbo-diimide and sodium azide lowered markedly the amount of toxins in the broth medium with no effect on mycelial growth. However mycelial extracts from cultures incubated in the presence of these metabolic inhibitors contained higher amounts of aflatoxins than did extracts from controls (Achmoody and Chipley, 1978).

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Aim of the Project

The foregoing review showed that the contamination of feedstuffs is a potential problem in all countries, specially those which import most of the feedstuff from tropical countries like India, Uganda and from countries where contamination by mycotoxin is an epidemic problem like U.S.A and Philippines. Since Jordan is no exception, this study was done to investigate the following :

1. To have an idea about the major types of animal feed used in Jordan.
2. To determine the incidence and amount of aflatoxins and sterigmatocystin in some feedstuffs.
3. To study occurrence of aflatoxin producing fungi in these feedstuffs.
4. To test the suitability of some feedstuffs as substrate for the production of aflatoxin.
5. To present an outlook on the situation concerning the use of these animal feedstuffs and to be aware of any problem involved.

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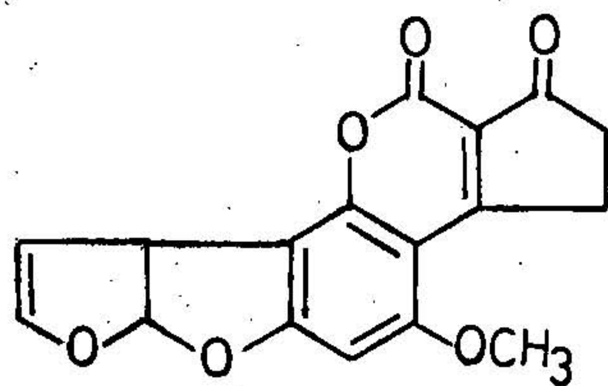

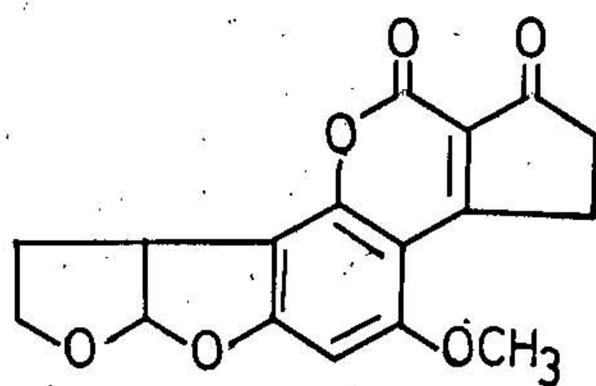

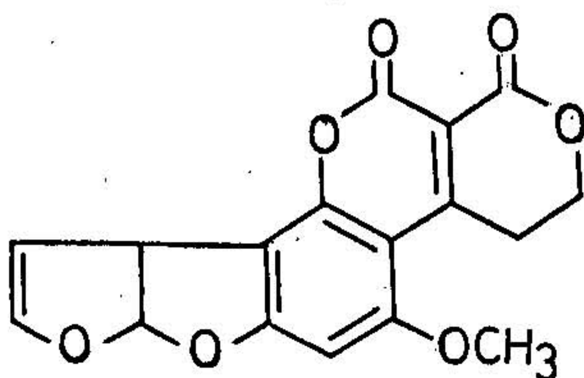

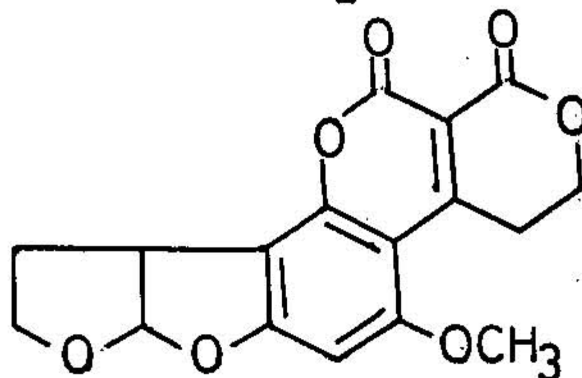

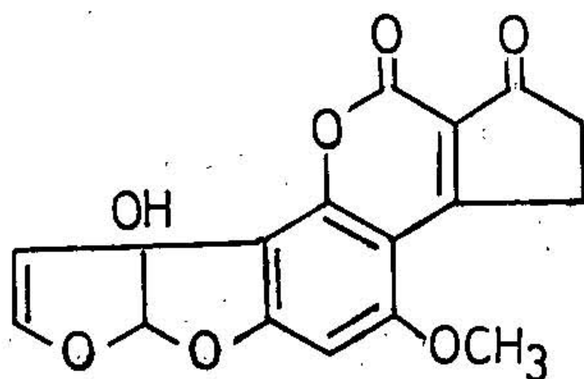

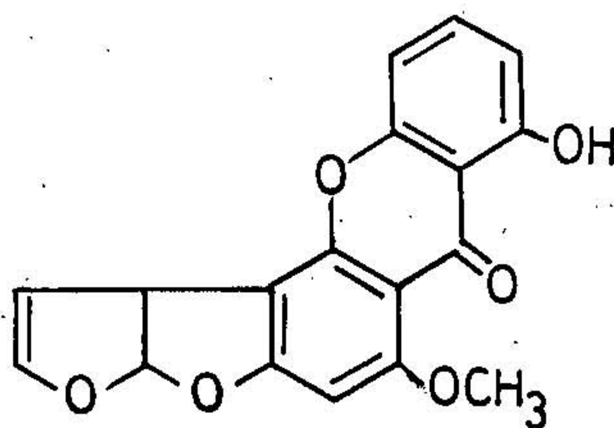

Fig.1. Structures of aflatoxins incriminated in food & feed contamination.

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Materials and Methods

Collection of Samples

Samples were collected during the period of February 1980 to March 1981 from different farms in Jordan. These included : Amman, Salt, Irbid, Azraq, Madaba, Karak, Dhlail, Jordan Valley, Mafraq, Jerash and Ajloun districts (Fig. 2). The samples were : Corn, soybean, barley, alfalfa, bran, poultry concentrate, mixed poultry feed, mixed cow feed, poultry pellets and lentils. Sample weight was ranging between 100 g and 1000 g. Sterile polyethelene bags were used to collect the samples from the farms and bring them to the laboratory. Each of these samples was used for toxin extraction as explained in the Figures below and for microbiological analysis, namely : isolation of aflatoxin producing fungi.

Isolation of aflatoxin producing fungi :

Samples were examined for the presence of aflatoxin producing fungi by two methods :

1. By culturing 0.8-1.0 gm of each sample on the surface of Czapek agar or Potatoes-dextrose agar (PDA). (as suggested by Prof. R. Natour)
2. By serial dilution of the samples.

0.1 ml of the suitable dilution was plated on Czapek agar or PDA agar plates. 3-4 plates were used

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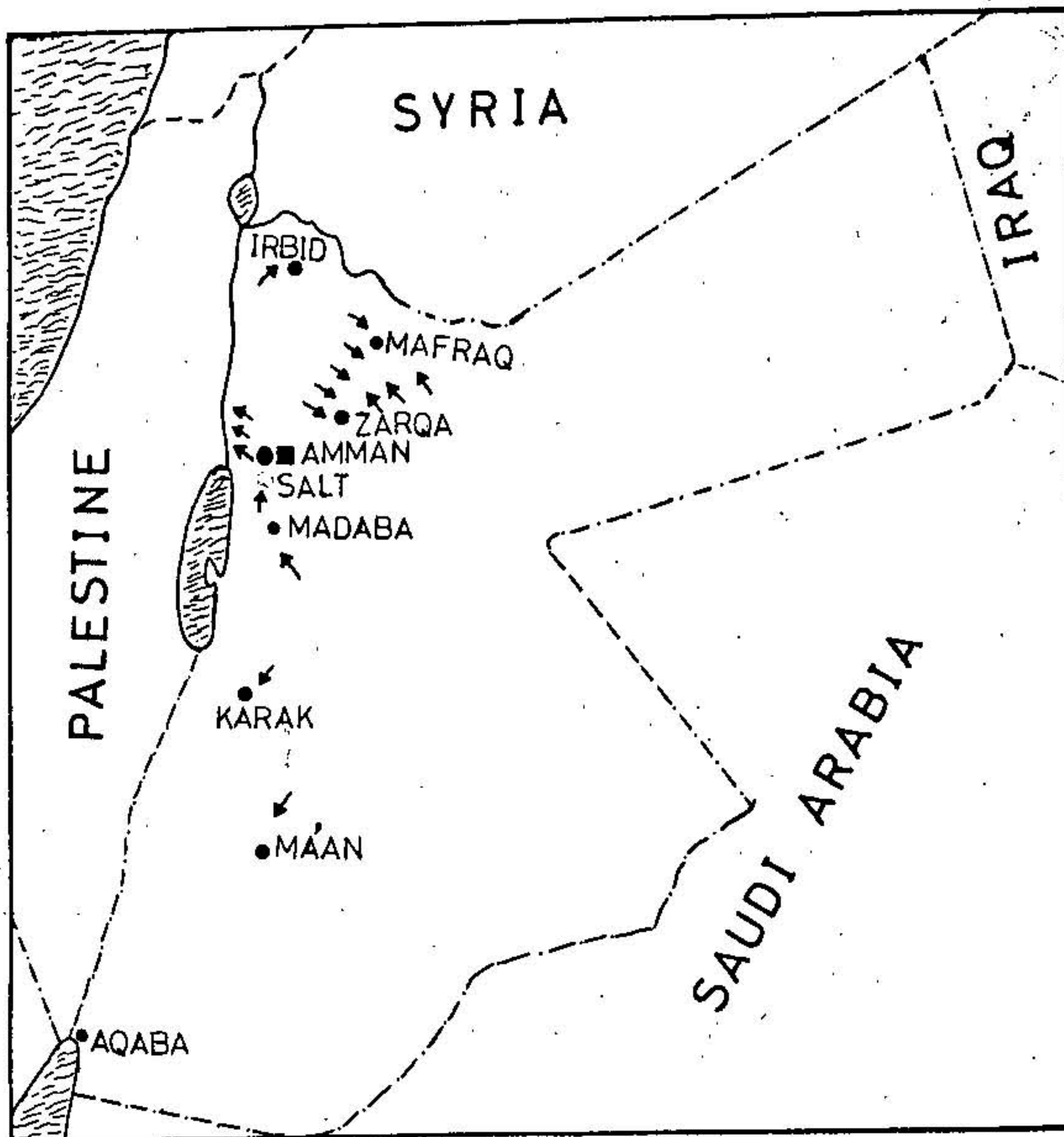

Fig.(2) _ Map of Jordan showing farming areas included in the study (↗) .

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for each sample. Then plates were incubated at 28$\pm$1^{o}C for 3-10 days. Fungal colonies, yellow to light green colors, suspected to be *Aspergillus flavus* group isolates, were transferred to Czapek agar plates. These pure isolates were slanted on screw cap tubes or small vials and then incubated for 7-10 days at 28$\pm$1°C until sporulation. Each isolate was examined microscopically to see the characteristic conidiophore, conidium, and conidiospores. Finally representative isolates were sent to the Commonwealth Mycological Institute (Kew, England) to confirm our indentification and to determine the strain of the isolate.

Aflatoxin Production by the suspected isolates :

Spores of each isolate were harvested from the screw-cap tubes by adding 1 ml of sterile distilled water to the culture and the slant was rotated for few minutes. Spore suspension (Ca 10^{7} spores/ml) was then poured into a 250 ml Erlenmeyer flask containing 100 ml of sterile laboratory aflatoxin producing medium of Salhab and Edwards (1976). The medium was incubated at 28$\pm$1°C in an orbital incubator running at 200 rpm for 5-9 days. Two laboratory aflatoxin-producing media were used to study the ability of the isolated fungi for production

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of aflatoxins. The one above and a medium described by Reddy *et al.*, (1971). The first medium (Salhab and Edwards, 1976) is composed of (per liter of distilled water) :

Glucose	50 grams
Ammonium sulfate $(NH_4)_2SO_4$	3 "
Dihydrogen potasium phosphate (KH_2PO_4)	10 "
Magnesium sulfate $(MgSO_4 \cdot 7H_2O)$	2 "
Ammonium molybdate $(NH_4)_6MO_{24}4H_2O)$	0.5 mg
Ferric sulfate $(Fe_2(SO_4)_3 \cdot 6H_2O)$	0.3 "
Copper sulfate $(CUSO_4 \cdot 5H_2O)$	0.3 "
Zinc sulfate $(ZnSO_4\ 7H_2O)$	2.96 gm
Boric acid $(H_3\ BO_3)$	1.0 mg
Manganese chloride $(MnCl_2)$	0.4 "

The medium was autoclaved at 121^oC and 15 PSI for 15 minutes before inoculation. The other medium that was used is the one described by Reddy *et al.*, (1971). This culture medium is composed of (per liter of distilled water) :

Sucrose	85 grams
Asparagin	10 "
Ammonium sulfate	3.5 "
Potassium dihydrogen phosphate	10 "
$MgSO_4 \cdot 7H_2O$	2 "

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$CaCl_2 \cdot 2H_2O$	10 grams
$ZnSO_4 \cdot 7H_2O$	10 "
$MnCl_2 \cdot 4H_2O$	5 mg
$FeSO_4 \cdot 7H_2O$	10 "
Ammonium molybdate $4H_2O$	2 "

The medium was autoclaved at 15 PSI for 15 minutes.

Fermentation :

One hundred ml of the suitable medium in 250 ml flasks was inoculated with 1 ml of spore suspension and incubated for 5-9 days in an orbital incubator running at $28 \pm 1^{\circ}C$ and 200 rpm. At the end of the incubation period, a considerable mycelial growth was obtained.

Aflatoxin extraction from the culture :

Aflatoxins were extracted from the broth medium by adding an equal volume of chloroform (100 ml). The flask contents were then blended by Waring blender at high speed for 3-4 minutes and filtered through three layers of cheese cloth. The extraction was carried out in a separatory funnel. The chloroform extracts (the lower layers) were pooled, concentrated under vacuum with flash evaporator at $40^{\circ}C$ and then evaporated to dryness under a nitrogen stream at room temperature. The

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residue was dissolved in 1 ml of chloroform for the purpose of purification-isolation and identification of the toxins. Fig. 3 shows the scheme followed to study the aflatoxin production by fungal isolates.

Aflatoxin extraction from feed samples :

In the first part of the study, the toxins were extracted from the samples by two methods depending on the nature of the sample. The two methods used were :

1. Method II (BF method) (AOAC, 26.02, 1975).
2. Seitz and Mohr Method (SM Method) : as described by Seitz and Mohr, 1972.

Method II (BF Method) (AOAC, 1975)

One hundred grams of the feed sample were added to 200 ml methanol water (55:45 v/v), 100 ml hexane and 4 gm of NaCl. This mixture was blended with a blender for about one minute at high speed. The homogenate was then centrifuged at 3000 rpm for 5 minutes. The supernatant was poured into a 125 ml separatory funnel and left for 10 minutes after which the aqueous methanol phase (at the bottom) was drained and an equal volume of

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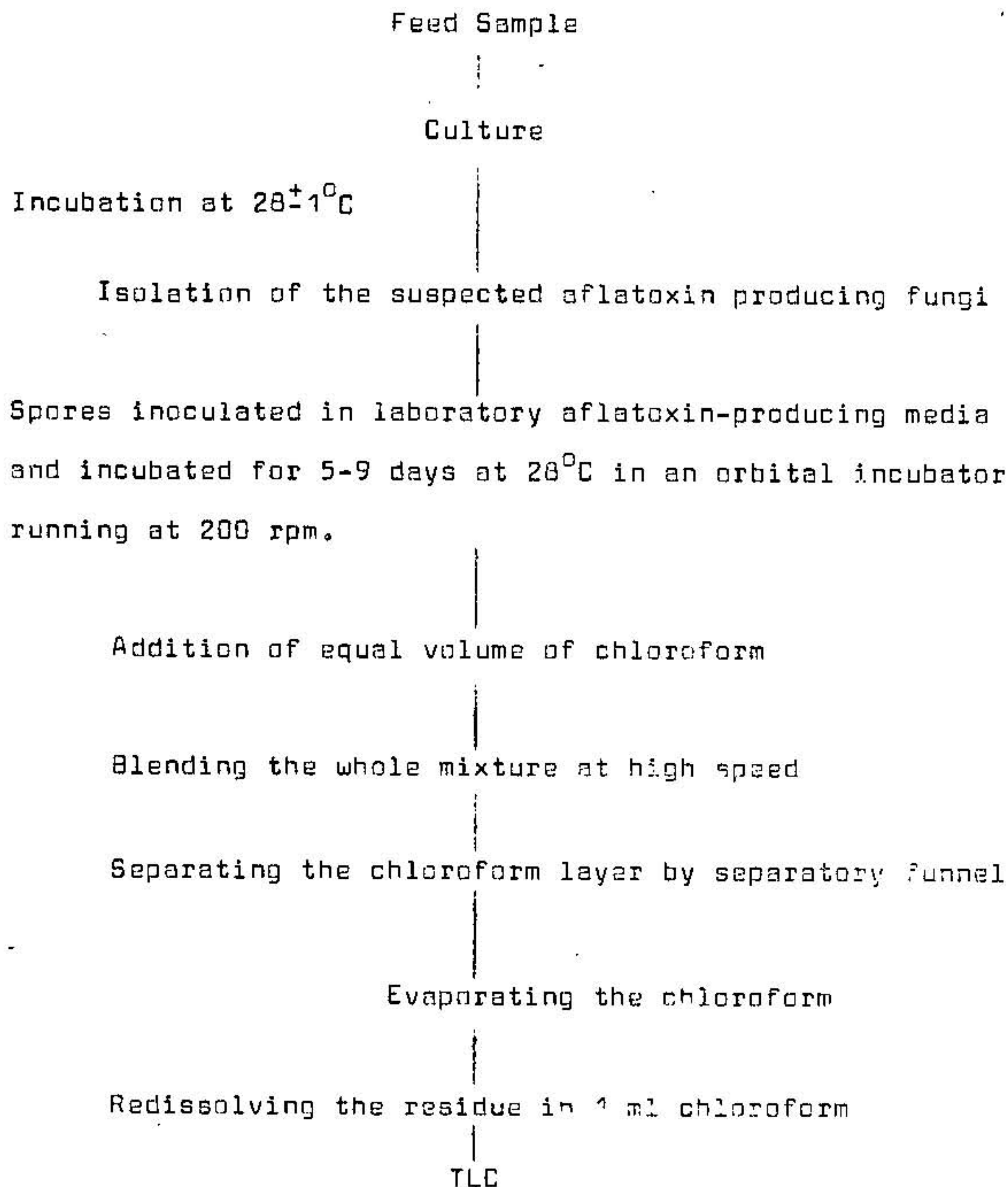

Fig. 3 : Scheme used for aflatoxin production by the fungal isolates and its extraction from the medium.

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Feed Sample 100 gm

MeOH: Water (55:45 v/v) 200 ml

n-Hexane 100 ml

NaCl 4 gm

Blending at high speed for one minute and centrifuged at 3000 rpm for 5 minutes and poured into separatory funnel

n-hexane layer (discard)

Shake the methanol : water layer for two minutes

add equal volume of chloroform

Shake the mixture for 1-2 minutes (several vents)

Drain the lower layer only

Chloroform

Evaporation

Clean up

T L C

Fig. 4 : The "BF" Method

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chloroform was added to this phase. This mixture was poured again into a clean separatory funnel and shaken for 30-60 seconds; then left for 15 minutes to separate completely in two layers. The bottom layer (chloroform) was drained into a flask and dried by rotary evaporator at 40-45°C. The residue was redissolved in 1-2 ml chloroform, poured into a 5 ml vial and stored in the freezer for further work such as cleaning up, isolation and identification of aflatoxin. Fig. 4 shows the scheme of the BF method.

Seitz and Mohr Method :

This method is recommended mainly for corn samples. Fifty gm of the feed samples and 100 ml methanol-water (75:25 v/v) were blended with waring blendor at high speed for two minutes. The mixture was centrifuged at 3000-rpm for three minutes. The methanol-water was poured into a separatory funnel to which a double volume (2:1 v/v) of 20% ammonium sulfate solution, and a volume of hexane, equal to the methanol-water volume; was added. The separatory funnel was stoppered and shaken for 60 seconds then left for 15 minutes, after which the hexane layer (upper layer) was drawn off. Twenty ml of methylene chloride were

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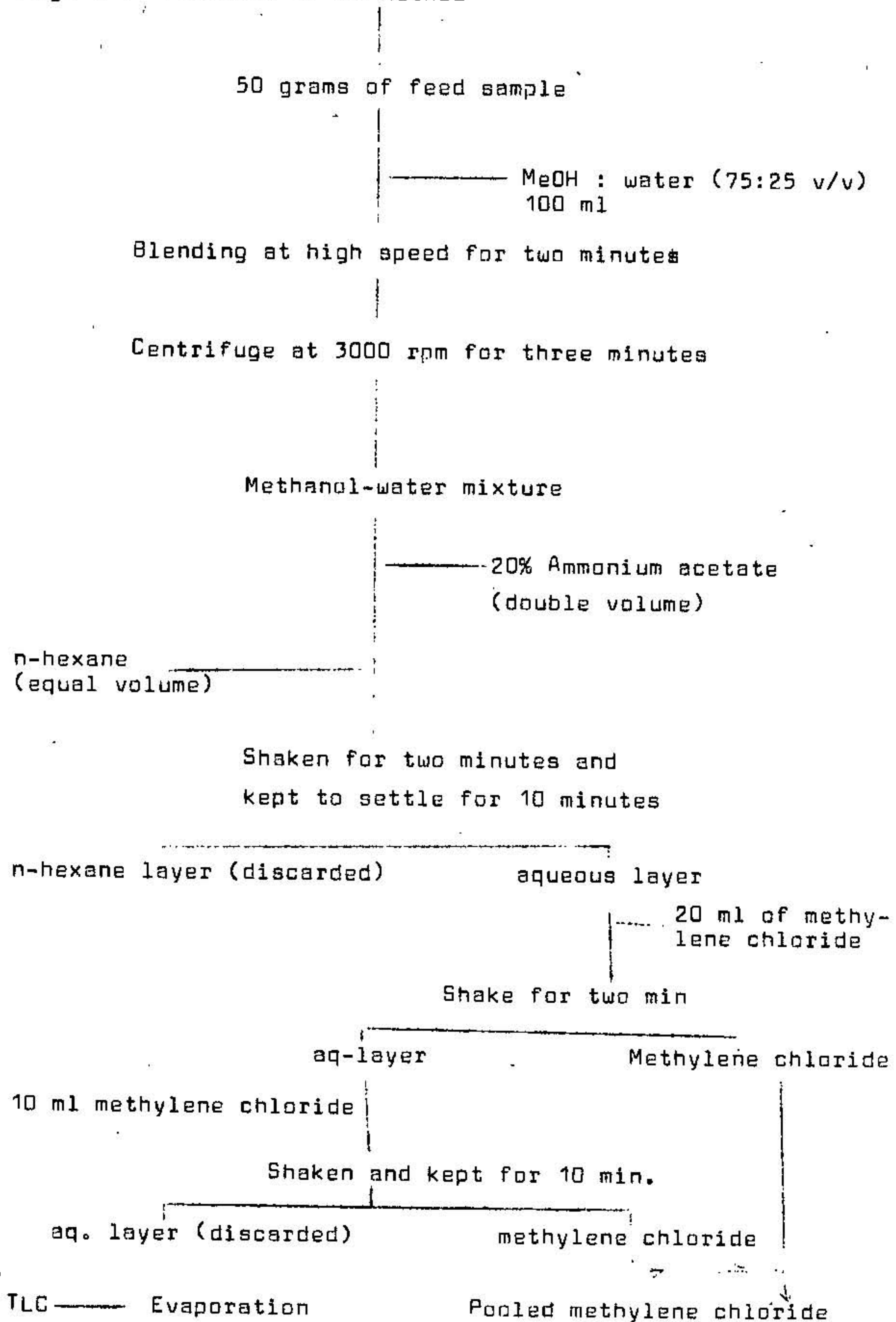

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added to the separatory funnel, shaken well and kept to settle down for 10 minutes, after which the methylene chloride (lower layer) was drained into a 50 ml flask. Another 10 ml of methylene chloride was added to the funnel, shaken well and left to settle for 10 minutes. The methylene chloride was drained into the flask containing the first portion, the pooled methylene chloride was evaporated to dryness under a stream of N_2 and after evaporation the residue was redissolved in 0.5 ml of chloroform to be spotted on thin layer chromatography plates. This method is quicker, easier, less expensive than the official method used by the AOAC for aflatoxin extraction from corn and shows similar results. The scheme is given in Fig. 5.

Extraction of aflatoxins and sterigmatocystin from feed samples :

The two following methods were recommended for extracting aflatoxins and sterigmatocystin at the same time, both methods were employed in this study.

Method I

This method was adopted by Salhab *et al.*, (1976) from the procedure outlined by Stack and Rodricks. The extraction was made from 50 grams of feed sample by

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blending it with 200 ml of acetonitrile-4% KCl solution (9:1 v/v) for 1-2 minutes at high speed and then kept to settle for a few minutes. One hundred ml of the upper phase (acetonitrile-water) was drawn and extracted with 50 ml hexane by separatory funnel. After that 50 ml of chloroform and 25 ml of dist. H_2O were added to aceto-nitrile layer and shaken well for one minute, kept to settle and separate into two phases. The acetonitrile-chloroform layer (lower layer) was drained into a flask and 25 ml of chloroform was added to the aqueous phase in the funnel, shaken well and kept to separate. The lower layer (chloroform) was drained to the previous flask, pooled and evaporated to dryness. The residue was redissolved in 1 ml of Benzene-acetonitrile mixture (98:2 v/v) and kept in a small vial for further analysis. Fig. 6 shows the scheme of this procedure.

Method II

This method was developed from procedures used earlier to extract aflatoxins from contaminated rice by Salhab et al., (1976). In this method 50 g of feed sample was mixed with 250 ml methanol chloroform (3:17 v/v) and this mixture was blended for two minutes at high speed and then filtered. The residual grain was blended again with 250 ml methanol : chloroform (3:17 v/v) for one minute, the filtrate was pooled and

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Method I (Stack & Rodricks, 1976)

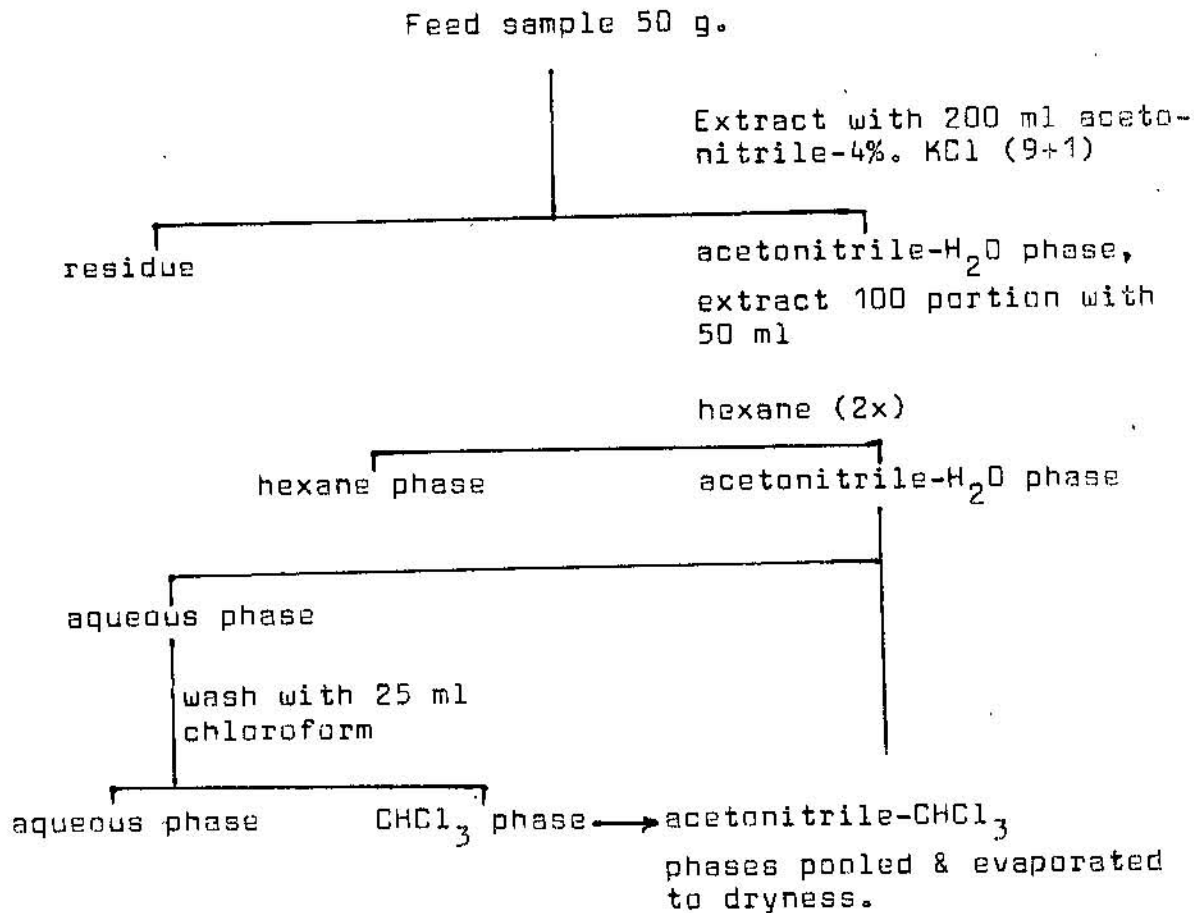

Fig. 6 : Scheme of Method I for extration of aflatoxin and sterigmatocystin from feed samples.

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Method II

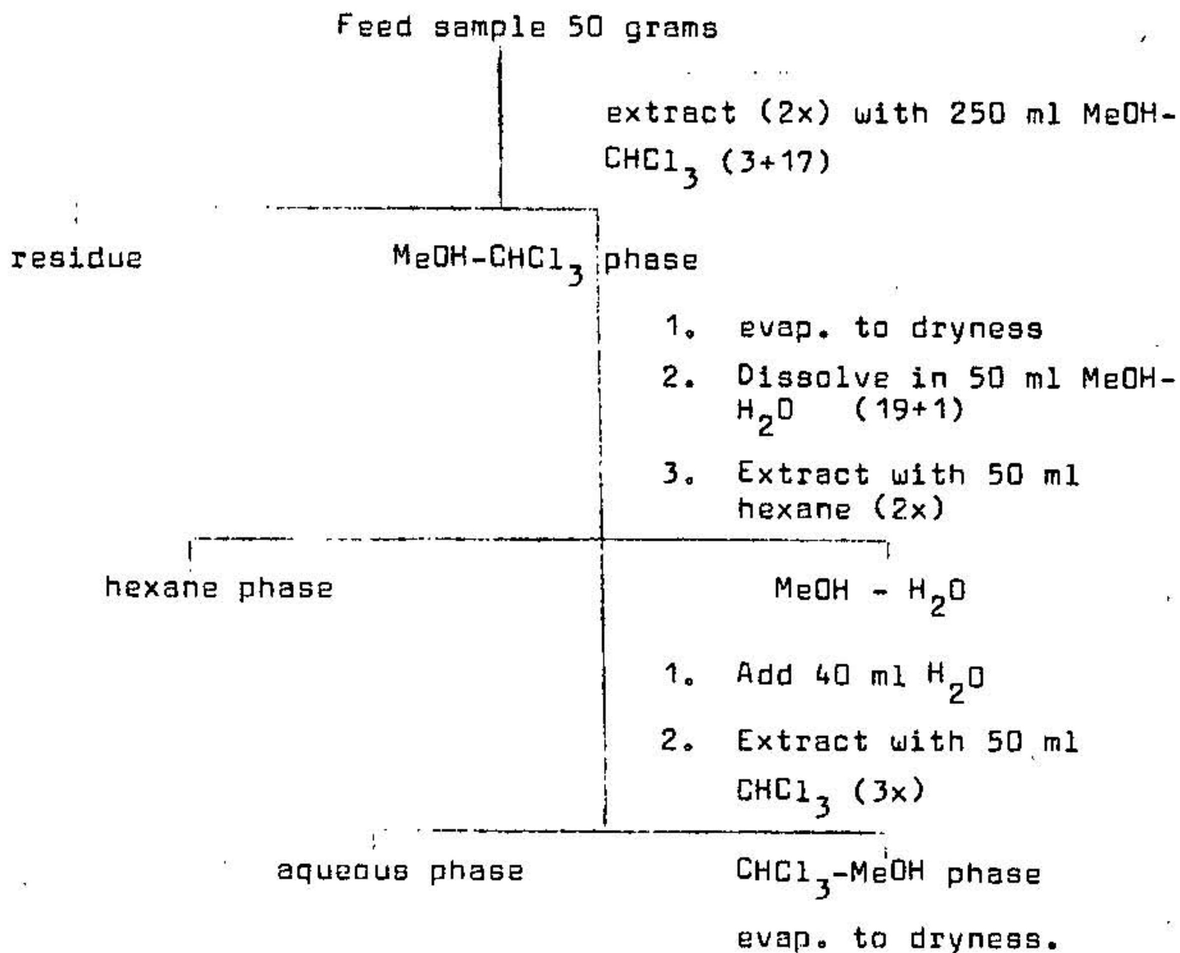

Fig. 7 : Scheme of Method II for extraction of aflatoxins and sterigmatocystin from feed samples.

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evaporated to dryness. The residue was dissolved in 50 ml methanol-water (19+1) and 50 ml portions of hexane were subsequently used to defat the extract. Forty ml distilled water was added to the methanol phase and this was extracted two times with 50 ml chloroform. Chloroform extracts were pooled and evaporated to dryness. The residue was dissolved in one ml of benzene-acetonitrile (98+2) and kept for further analysis. Fig. 7 shows the scheme of this procedure.

Thin layer chromatography for separation and identification of aflatoxins and sterigmatocystin

Silica Gel or, Adsorbil-1; was spread on 20x20 cm plates at a thickness of 0.25 mm. The plates were dried at laboratory conditions for 15 minutes and then activated at 105°C for one hour. The plates were used directly after cooling or stored in the desiccator for later use. Aliquotes (100 µl) of the sample extract dissolved in chloroform or benzene-acetonitrile were applied as a spot approximately 3 cm from the botton of the plate. The spotted TLC plates were first developed with diethylether and then with either chloroform-acetone (9:1 v/v), if only aflatoxins were suspected; or with toluene-ethylacetate-acetone (3+2+1), if

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only aflatoxins were suspected; or with toluene-ethylacetate-acetone (3+2+1), if aflatoxins and sterigmatocystin are suspected. This provides a relatively pure aflatoxin and sterigmatocystin preparation, as most of the contaminating pigments were carried close to the solvent front by the diethyl ether, while the toxins migrate with the second solvent. Aflatoxins and sterigmatocystin were identified on the basis of comigration with standard toxins and by their characteristic colors under long UV-light. To confirm the identity of sterigmatocystin, the plate was sprayed by 4% KCl in absolute methanol, the color of sterigmatocystin turns from brick-red fluorescence to yellowish (yellow-green) color. For the purification of the whole organic extract, one ml of chloroform or benzen-acetonitrile (98+2) was streaked on plates and developed with the solvent systems as described above. The fluorescent aflatoxins bands were scraped off and extracted from the silica by chloroform. Small amounts of the scraped powders were resuspended in 2-3 ml of absolute methanol for spectophotometric scanning. The screaning was recorded from 200 to 400 nm using Hitachi 200 spectrophotometer. The spectra were compared to that of the standard aflatoxin.

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Determination of the aflatoxin concentration :

The concentration of aflatoxins was determined by measuring the absorbance of the extract at a wave length of 360 nm. The concentration was calculated according to the following equation (AOAC,26.009; Masri, et al., 1968)

$$\text{ugm aflatoxin/ml} = \frac{A \times MW \times 100 \times CF}{E}$$

CF = Correction factor (dependant on the spectrophotometer used)

MW = Molecular weight of aflatoxin

A = Absorbance

E = Molar absorption (AFB_1, 19,800, B_2 20,900, G_1 18,200)

Suitability test for aflatoxin production :

Some feedstuffs used frequently in Jordan, were tested for their suitability as substrates for aflatoxin production. These feed samples were : poultry concentrate, bran, poultry pellets and lentils. Corn was used as control. The method used for this purpose was developed by Shotwell *et al.*, 1966.

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Culture and inoculum :

Aspergillus parasiticus NRRL 2999, is one of the best known aflatoxin-producing fungi. Fresh slants were prepared from the stored strain, incubated at $28\pm1^{o}C$ for 7-10 days (until sporulation). Spores were harvested by adding 5 ml of sterilised distilled water, slants were shaken to give a uniform suspension of spores. One ml suspension containing about 10^7 spores/ml was used to inoculate 50 grams of the substrate.

Fermentation :

Fifty grams of the feed samples were added to 25 ml distilled water in 500 ml Erlenmeyer flask. The mixture was shaken for two hours, then autoclaved and inoculated with about 10^7 spores of *A. parasiticus* NRRL 2999. The flasks were allowed to stand for one hour, then placed in an orbital incubator running at $28^{o}C$ and 200 rpm. The flasks were watched daily, and small amounts of sterile distilled water were added at different times to prevent clumping. The flasks were occasionally removed from the shaker and shaken vigorously by hand whenever clumping was observed. The growth of fungi was observed by naked eyes. At the time of harvest,

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after 8-12 days; chloroform was added (150-200 ml) to the culture and it was kept in the refrigerator to be analyzed within 36-48 hour.

Assay Procedure

The sample with cloroform was blended for five minutes at high speed. The mixture was centrifuged for 10 minutes at 2,000 rpm and filtered into a separatory funnel. The chloroform layer was passed through anhydrous sodium sulfate to remove traces of water. The filtrate was evaporated to dryness as described earlier and the residue was dissolved in 2 ml chloroform for further purification and quantitation. The scheme of the Shotwell-procedure used for this purpose is presented in Fig. 8.

Confirmatory tests for aflatoxins :

To confirm the identification of the isolated toxin. A portion of it was treated separately with trifluoroacetic acid and acetic thionylchloride. Aliquots of the reaction product, together with standard AFB_1 and AFB_2 treated similarly; were spotted on TLC. After development of the plate, the Rfs of the spots were compared under ultra-violet light.

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Moisture content determination

The moisture content of the sample was determined by drying 10 gms of the samples at $90^{o}C$ for 48 hr. (Al-Adil *et al.*, 1976).

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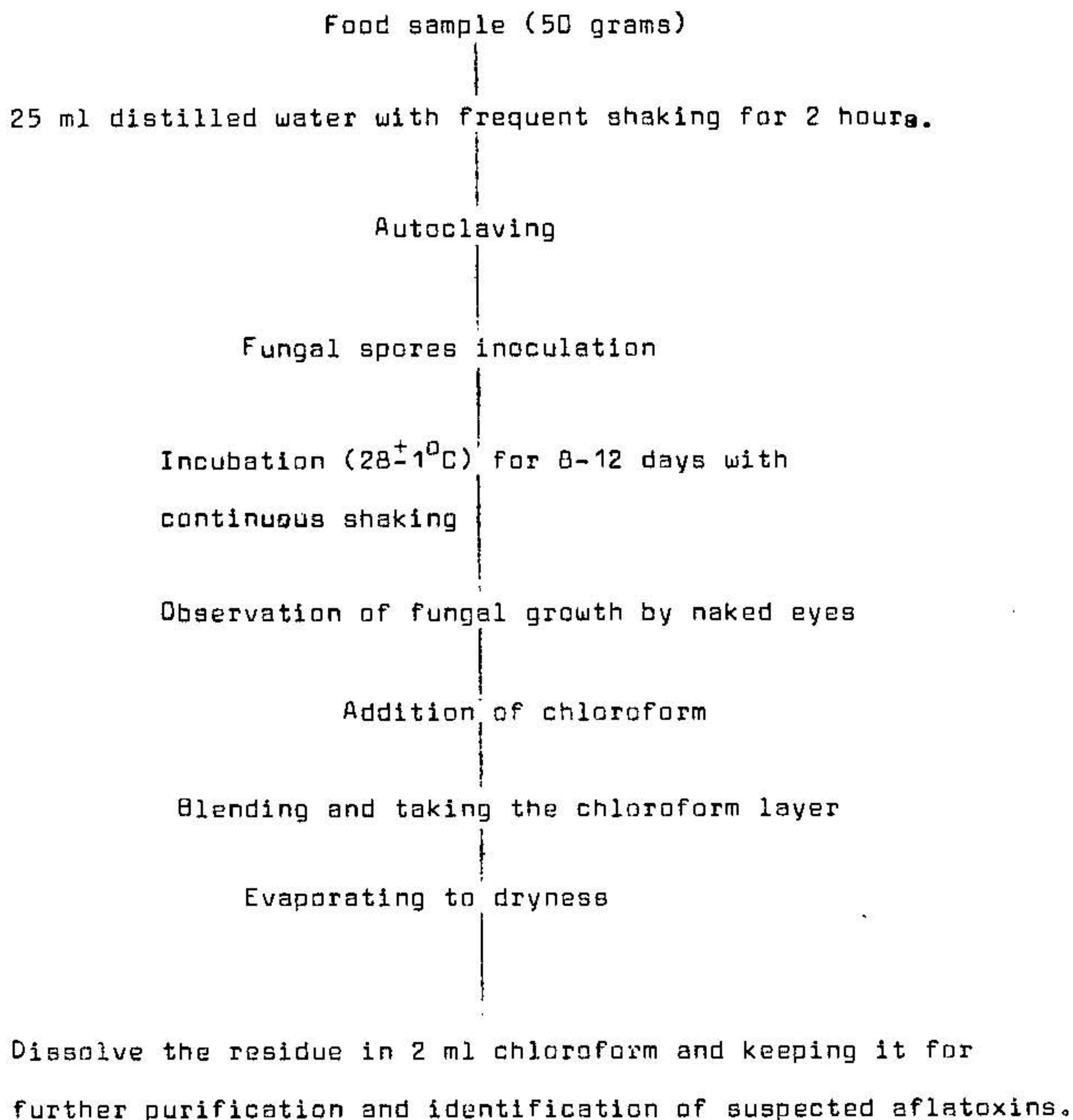

Fig. 8 : Scheme used to determine the suitability of certain substrate for aflatoxin production.

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RESULTS

Occurrence of aflatoxins, sterigmatocystin and their levels of feed samples

Six hundred and six samples of feedstuffs were collected from different farms in Jordan as indicated in Fig. 2. All these samples were tested for the presence of aflatoxin, 301 samples were tested for sterigmatocystin and 575 samples were also cultured to isolate *Aspergillus flavus* group fungi. These samples, as shown in Tables 1 & 2; were : 91 corn samples, 94 soybean, 83 poultry concentrate, 105 mixed poultry feed (this is a mixture of corn, soybean, poultry concentrate extracted from organic sources like fish, with some salts and sometimes some essential aminoacids are added), 29 mixed cow feed (this is a mixture of barley, soybean, bran and some salts), 46 bran, used mainly as cow feed and in some poultry farm 66 barley, (used as cows and sheep feed), 37 poultry pellets, this is given to poultry after a certain age (40-45 days), and is similar in its constituents to the fine mixed poultry feed, 13 lentil found in few cows and poultry farms, used at hot weather, and 42 alfalfa from cow farms in Deir-alla farming center,

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Wadi Al Dhlail farming center, and from Jabri-cows farm in Russayfeh.

Only one corn sample out of 91 (1.1%) was found to contain naturally AFB_1 and AFB_2. All other samples were free from aflatoxins and sterigmatocystin.

Moisture content determination :

The moisture content of the different samples was found as listed in Table 3.

Although the moisture content in all the samples was less than the optimal moisture content (18%) (Ciegler, 1977) for aflatoxin production. It still was higher than the minimal moisture content required for aflatoxin production (10%) (O'Brien, 1976).

Contamination of feed with Aflatoxin-Producing Fungi :

The presence of aflatoxins in the tested samples was very low (0.16%). Meanwhile the fungal isolates of A. flavus group, which were suspected to produce toxins; were detected in 41.4% of the samples tested (Table 4). These isolates were tested for aflatoxin

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Table 1. : Number of different feed samples for aflatoxin (AF), sterigmatocystin (St), and *A. flavus* group fungi.

Feedstuff	No. of samples tested for aflatoxin	No. of samples tested for sterigmatocystin	No. of samples tested for *A. flavus* group
Corn	91	46	88
Soybean	94	48	90
Poultry concentrate	83	38	75
Mixed poultry feed	105	51	104
Mixed cow feed	29	13	27
Bran	46	22	44
Barley	66	30	66
Poultry pellets	37	15	33
Lentil	13	8	11
Alfalfa	42	13	37
Total	606	301	575

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Table 2 : Description of feed samples collected and the frequency of its use as animal feed

Type of feed used	No. of samples included in the study	Frequency of its use	Description	Animals feeding on such feed
Corn	91	very frequently	Intact or homogenized good quality, no damage.	Poultry, occasionally cows
Soybean	94	-ditto-	Homogenized, no visible contamination.	Poultry, and cows
Poultry concentrate	83	frequently	Mixture of organic materials dark brown color, homogenized Mostley fish.	Poultry
Mixed poultry feed	105	very frequently	Mixture of corn, soybean, concentrate, prepared in the farm or oftenly in the factories	Poultry
Mixed cow feed	29	frequently	Mixture of barley soybean and bran.	Cows & sheep
Bran	46	sometimes	Good quality, no contaminants, from wheat	Cows & some poultry
Barley	66	very frequently	Good quality, no damage, given homogenized	Cows
Poultry pellets	37	Not common	Composed of corn, soybean, concentrate, corse	Poultry
Lentil	13	Rarely	Poor quality, red color crushed	Poultry & cows
Alfalfa	42	frequently	Varies from wet green to dry yellow, tightly packed	Cows

production on synthetic media. Only 22 (9.2%) of the suspected isolates were found to be producers for one or more of the aflatoxins (Table 5). These aflatoxigenic isolates were present with varying proportions in all types of feed tested with the exception of lentil and soybean (which were found to be free from aflatoxigenic isolates). The highest occurrence of aflatoxigenic isolates was found in mixed cow feed and bran (13.3%), this is probably due to the fact that such feed provides a better growth media for the fungal spores. The lowest occurrence was in poultry concentrate and barley (6.6%), while other samples were as follows (Table 5) : Corn (9.4%), mixed poultry feed (10.1%), poultry pellets (12.5%) and in alfalfa (11.1%). The isolates were found to produce one or more types of AF (Table 6). Representative isolates of the fungi were sent to the Common-wealth Mycological Institute (CMI) in Kew (England) for further identification. The results indicated that isolates were *Aspergillus flavus* link ex fries, *Aspergillus parasiticus* spear which are the natural aflatoxin producers in food and feedstuffs (Goldblatt, 1969). Other isolates were identified also as *Aspergillus tamari* and this strain produced AFB_2 on laboratory medium, and as *Aspergillus oryzae* and this isolate produced AFB_1. These two isolates, *A. tamari* and *A. oryzae*; are not known to be natural aflatoxin

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Table 3. Moisture content of feed samples

Feed sample	No. of samples tested	Moisture content (%)		
		Range		Mean
Corn	63	11.0	11.9	11.4
Soybean	69	11.1	12.2	11.6
Poultry concentrate	58	10.8	11.4	11.0
Mixed poultry feed	93	10.4	12.1	11.7
Mixed cow feed	23	11.2	12.2	11.9
Wheat bran	37	13.4	16.7	15.0
Poultry pellets	34	11.6	12.8	11.8
Lentil	9	10.7	12.5	11.9
Alfalfa	27	10.8	18.7	15.0
Barley	55	12.0	13.20	12.8

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Table 4. : Number and percentage of suspected *A. flavus* isolates from each type of feed

Feedstuff	No. of samples cultured	No. of suspected isolates	% of suspected isolates
Corn	88	53	60.2
Soybean	90	11	12.2
Poultry concentrate	75	15	20.0
Mixed Poultry feed	104	69	66.3
Mixed cow feed	27	15	55.5
Wheat bran	44	15	34.0
Barley	66	30	45.4
Poultry pellets	33	8	24.2
Lentil	11	4	36.3
Alfalfa	37	18	48.6

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Table 5 : Number of suspected isolates and the percentage of those capable of producing aflatoxin under laboratory conditions

Feedstuff	No. of suspected isolates	No. of toxigenic isolates	% of toxigenic isolates
Corn	53	5	9.4
Soybean	11	0	0.0
Poultry concentrate	15	1	6.6
Mixed poultry feed	69	7	10.1
Mixed cow feed	15	2	13.3
Bran	15	2	13.3
Barley	30	2	6.6
Poultry pellets	8	1	12.5
Lentil	4	0	0.0
Alfalfa	18	2	11.1
Total	238	22	9.2

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Table 6 : Types of toxins produced by isolates from different feed samples

Feedstuff	No. of toxigenic isolates	Types of toxins produced	No. of producer isolates
Corn	5	B_1 & B_2	3
		B_1	1
		B_2	1
Poultry concentrate	1	B_2	1
Mixed poultry feed	7	B_1, B_2	1
		B_1, B_2&G_1	3
		B_1	2
		B_2	1
Mixed cow feed	2	B_1	2
Bran	2	B_1	2
Barley	2	B_1 & B_2	1
		B_1	1
Poultry pellete	1	B_2	1
Alfalfa	2	B_1	2
	22		

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producers in natural substrate but implicated in aflatoxin production under laboratory conditions.

The suitability of some feedstuffs for aflatoxin production under laboratory conditions

Most of the feedstuff samples used in Jordan are imported and used internationally. Most of them have been tested for aflatoxin production in other laboratories (Goldblatt, 1969). However, some feedstuff, like poultry concentrate, poultry pellet, wheat bran and lentil were tested as presented in Tables 7 & 8.

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Table 7 : Suitability of few feedstuffs for aflatoxin production

Foodstuff	Fungal growth	Aflatoxin production
Poultry concentrate	++	Positive
Poultry pellets	+++, S*	"
Bran	++	"
Lentil	++	"

* S = Heavy sporulation

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Table 8 : Amounts and types of aflatoxins produced by *A. parasiticus* NRRL 2999 inoculated to certain feedstuffs

Feedstuff	Aflatoxin types & concentration µg/gm substrate			
	AFB_1	AFB_2	AFG_1	AFG_2
Poultry concentrate	18	5	1.4	-
Poultry pellets	63	11	8	0.4
Bran	34	9	7.5	-
Lentil	18	4.5	6.5	-
Corn*	70	11	7	1

* Corn was used as a reference sample

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DISCUSSION

Much of the justification for research and education in mycotoxicology resides in the occurrence of Mycotoxicosis as a source of potential hazards for the health of man and animals, and as an economic factor in the animal and feed industries. Under the influence of this threat, together with other previously mentioned factors; this study was conducted. It covers a wide range of feedstuffs used in Jordan. Among ten different feedstuffs covered in this study (Table 1), aflatoxins as such were confirmed in only one type, the corn, where 0.16% of the tested samples were found to be contaminated naturally with these toxins. This rate of aflatoxin presence compaired with reported findings in other countries from similar studies, is considered very low. For example, in Canada, Funnell _et al._, (1979) studying different types of animal feedstuffs. They reported the presence of one or more of different mycotoxins (aflatoxins, sterigmatocystin, ochratoxin, patsulin, zeralenone) in 13.7% of the feedstuffs. However, the amounts of toxins, found were low and sterigmatocystin was absent from most of the samples. In Australia, Bryden _et al._, (1980) found AFB_1 in 41% out of 55 samples of

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different feedstuffs. They tried to correlate field outbreaks of animal disease in farms from where the samples were collected. They were able also to isolate the toxins from animals. Although this is one of the highest reported cases of aflatoxin contamination it might be due to the small size of the samples.

Similar studies were conducted in foods and feeds where higher rates of aflatoxin incidence were reported. In Poland 8.7% of 150 samples examined yielded aflatoxins (Juskiewica, 1977). In Egypt aflatoxins were found in 33% of 42 samples (Girgis et al., 1977) and in New Zealand 5.3% of 38 samples of different fee[illegible] were aflatoxin positive (Staton, 1977). Diet components obtained from a West German manufacturer of laboratory animal diets were analyzed for aflatoxins by Obi, (1978). Of the 16 components examined 6 (37.5%) contained detectable levels of AFB_1.

In a recent study done by Jarrar, 1980 in Jordan, it was found that 1.2% of the foodstuffs samples were naturally contaminated with aflatoxins.

In this study, the amounts of aflatoxins found in the corn samples (AFB_1; 33 ppb, AFB_2; 12 ppb)

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represents a dangerous level, knowing that one part per billion in the diet produced liver cancer in trout in a year and 8-20 ppb induced hepatoma in 4-6 months in the same animal (Lee et al., 1971). These levels are also high if we compare it with that found in corn tested by Shotwell et al., (1977) where 24-47 ppb of AFB_1 were recorded (and up to 209 ppb in some samples).

The types of feed used in Jordan, except corn; appear to be from those commodities that are known not to be good natural substrates for aflatoxin production like soybeans, barley, lentil and wheat bran (Llewellyn & O' Rear, 1977), while several incidences of aflatoxins in corn were reported (O'Brien, 1976, Goldblatt, 1969, and Zubber, et al., 1976) and it was classified as a suitable substrate for natural production of aflatoxin (Heathcote & Hibbert, 1978). The rate of aflatoxin presence in corn in this study was (1.09%), one out of 91 samples tested, this rate is low compared with that reported by some investigators. Shorwell et al., (1970) found that 2.12% of 283 corn samples tested were found to contain in Southeastern United States (Zubber et al., 1976) revealed a much higher incidence (49%) of aflatoxin-contamination. It seems that the rate of corn contamination by aflatoxins is very variable, this is clear

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from studies done in the United States at 3 successive years where the rates of corn contamination were 13%, 27% and 52% in 1972, 1973 and 1974, respectively as presented by Zubber et al., (1976). However results similar to the results in this report were obtained by Hesseltine et al. in 1964 and 1965. They examined over 1,000 corn samples for aflatoxins and only small amounts of aflatoxins were detected in a number of low-grade samples. The corn that is used as feedstuff in Jordan is generally of very good quality and this is clear from the intact undamaged seeds since it is noticed that a direct relationship exist between damage (mechanical or insect), molds infection and toxin production (Goldblatt, 1969 & Shotwell et al., 1977). The moisture content of the corn being about (11.4%) is low and far from promoting aflatoxin production, specially in this good, undamaged quality.

Soybean samples were free from aflatoxins. This result is in agreement with the results of other studies which indicated beans to be poor substrate for aflatoxin production (Gupta and Venkitasubramanian, 1975). Shotwell et al., (1978) surveyed 1046 samples of soybeans for the presence of aflatoxins, only 2 samples showed aflatoxins and at a low level. Barley used as animal feed in Jordan is of good quality. Also, pure and no visible

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contamination could be detected. Hoower, barley and other small grains are not a dangerous source of aflatoxin contamination (Ciegler, 1977). No aflatoxin or sterigmatocystin were isolated from any of the samples tested in this study.

Mixed poultry feed and poultry pellets, which are mixtures of corn, soybeans, and poultry concentrates, were found to be free from aflatoxins and sterigmatocystin, probably because of low moisture content together with the good quality of the ingredients.

The occurrence of aflatoxin producing fungi in different feedstuffs

Toxins were found in corn only but the molds that produce the aflatoxins, *Aspergillus flavus* group fungi, were isolated from 8 types i.e., (80%) of the different feddstuffs tested. The rate of contamination by aflatoxigenic isolates was highest (13.3%) in wheat bran and mixed cow feed, although the samples were free from the toxin itself. Isolates of *A. flavus* group were found in 53 sample of 88 corn samples cultured (60.2%). Comparing this rate (60.2%) with similar studies (Goldblatt, 1969) where *A. flavus* contamination was 75.4%, reveals that

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the rate of contamination by *A. flavus* group is reasonable. However, higher rates were reported by Hesseltine *et al.*, 1975 where *A. flavus* group were isolated from more than 99% of 1283 corn samples tested. Since we can not differentiate between isolates that are toxin producers and non toxin producers from the morphology or microscopic examinations and since several investigators have indicated that only some strains of *A. flavus* group were capable of aflatoxin formation (Sargent *et al.*, 1961 and Hesseltine *et al.*, 1968), all of the isolates were tested for their ability to produce aflatoxin on an appropriate medium. The results showed that five out of 53 *A. flavus* isolates, obtained from corn; were found to be toxin producers.

Ninety samples of soybeans were also cultured in search of *A. flavus* group and none of the eleven isolates were able to produce aflatoxin under experimental laboratory conditions.

This investigation indicates that 22 (9.2%) of all the isolates (238) were toxin producers. Similar studies in other countries showed variable rates of toxigenic isolates. In Japan 25.8% of *A. flavus* isolates, from different food & feeds; were aflatoxin-producers and eleven of nineteen (57.9%) of A. *versicolor* isolates

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sterigmatocystin producers (Takatori et al., 1980). Afzal et al., (1979) from Pakistan, cultured 54 samples of different feedstuffs, 21 isolates of *A. flavus* (38.9%) were obtained, only one isolate (4.7%) of these was toxin producer and was identified as *A. parasiticus*. Stoloff et al. (1976) found that 10.6% of the isolates were toxin producers. In Russia, 12% of the isolates obtained by L'vova et al. (1978) were aflatoxin producers. In India, only 6% was reported by Rao et al. (1965). Studies in the neighbouring countries on food and feedstuff indicate rates of incidence of toxigenic isolate close to the rate found in this study (9.2%). In Palestine 20% and 12.7% in 1963 and 1964, respectively; were reported (Brout and Joffe, 1966). In Iraq and in a study on some food and feedstuffs, 7.2% of the isolates were toxin producers. In Jordan as reported by Jarrar 1980, 14.2% of 219 fungal isolates from food-stuffs were toxin producers. Higher rates of toxigenic isolates were found in areas where high rate of aflatoxicosis was observed as some African countries when 52% of the isolates from food or feed were found to be aflatoxin producers (Austwich and Agerst, 1963).

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Suitability of some feedstuffs for aflatoxin production under laboratory conditions :

Four different feedstuffs were tested to find out whether they are suitable to produce aflatoxins or not. Corn, known to be a good substrate for aflatoxin production (Goldblatt, 1969, O'Brien, 1976), was used as control in this study.

Amounts of aflatoxin produced on poultry concentrate were 18,5, and 14 μ/gm of AFB_1, AFB_2 & AFG_1, respectively. The amounts produced on corn, used as control, were 70, 11, 7 and 1 μg/gm for AFB_1, AFB_2, AFG_1 and AFG_2, respectively. Rice, under the same conditions, produced 100, 7, 11 μg/gm of rice of AFB_1, AFB_2 and AFG_1, while peanut produced 76, 13, 6 μg/gm of AFB_1, AFB_2 and AFG_1, respectively.

These results reveal that the poultry concentrate is, potentially, suitable for aflatoxin production. The danger of this feedstuff can be visualized if we know that it is given in all poultry farms in Jordan. This danger becomes greater in egg-producing farms, where the birds live for about 30 months, so small amounts of toxin is enough to be a source of hazard.

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Poultry pellet was also positive for aflatoxins. This was expected since corn constitute about 60% of the mixture. Soybean (representing about 30% of the pellet weight) also showed the ability to produce aflatoxins, but at low level (Hesseltine et al., 1966; Shotwell et al., 1978). The pellet form of this feedstuffs showed the ability to promote heavy growth and sporulation of the fungus, a primary step in aflatoxin production. The amounts produced when the pellet was used as substrate for NRLL 2999 amounted to 63, 11, 8 and 0.4 ug/gm of AFB_1, AFB_2, AFG_1 and AFG_2 respectively. Comparing these amounts with those produced on peanut, rice and corn, in this study and under similar conditions, we can classify this mixture as a good substrate or as good as peanut and corn for aflatoxin production. However, this pellet form was found to be used in few farms for adult birds.

Lentils, a minor element in animal feed in Jordan; was found to be a suitable substrate for aflatoxin production also. Table 8 shows that the amounts of aflatoxins produced on lentil are less than those produced on poultry concentrate, pellets and bran. Lentil was found to be used rarely in few farms. However lentil is used as human food in our country

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and the neighboring countries. The storage practice of lentil in the house for a year or so; is still a common habit and might pose a source of danger on public health.

Wheat bran is used in cow farms and some poultry farms. The amounts of aflatoxins produced, as in Table 8, classify it as a good substrate for aflatoxin production, however the samples tested in this study were of good quality except for few samples which were heavily contaminated with Penicillium spp. The dangerous alarm of bran usage as feed can be seen if we know that it is given for dairy cattle almost all over the year, except few months of spring. And if little amounts are consumed, it will be secrated in the milk as "milk toxin" (de Iong and van Pelt, 1964). The transfer of aflatoxin to milk product, such as cheese and yogort, is under study by Applebaum et al. (1979). Their primary results showed the presence of toxin in cheese.

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CONCLUSIONS

This study showed that :

1. All feedstuffs, used in Jordan; are potentially suitable for aflatoxin production. So if these substrates are infected by toxigenic isolates; toxin will be produced. However, corn is cited in the literature to be a world wide source of aflatoxin contamination, while other feedstuffs, used in Jordan; like soybeans, bran, poultry concentrate barley, are not dangerous sources for contamination.

   Nuts, in general, are not used in animal feeding in Jordan, and this eliminates a lot of hazards, since nuts are known to be the best substrate for aflatoxin production.

2. *Aspergillus flavus* group fungi, the natural producers of aflatoxins, are common in feedstuffs and the percentage of toxigenic isolates is comparable with that found in other countries.

3. The moisture content of feedstuffs found in the samples included in this study is, generally; less than the preferable moisture content desired by the mold for growth and production of toxins. As a result, it should be stressed upon the storage practices of such

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material to prevent any further rise of moisture content which will result in improved growth and toxin production by the fungi.

4. The temperature, specially in localities where animal farms are concentrated like Al Ghor and Edhlail, promotes the growth and the production of aflatoxins in long summer season, this also should be controlled as indicated above.
5. Long lasting storage of feeds is, generally, not common in Jordan, especially in farms. Many farms discard old feeds or the left over of each patch of feed for the sake of the health of animals, i.e. to prevent the infection of the new patchs of animals.
6. Animal feeds in Jordan are usually imported from different countries. This depends upon commercial aspects. So the results of this study throw light on the problem of animal feed contamination during the period of the study, i.e. February 1980-March 1981.
7. These results are not an alternative of ever-lasting control on food and feedstuffs; imported or produced locally.
8. Countries aware of aflatoxicosis problems established special laboratories at their sea-ports to test the imported foods and feeds that are reported to be suitable for aflatoxin contamination, so we recommend interested authorities to follow this example.

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بسم الله الرحمن الرحيم

تواجد سموم الافلاتوكسن والفطريات المنتجة لها في أغذية الحيوانات المستعملة في الأردن

شملت هذه الدراسة ستمائة عينات من عشرة أنواع مختلفة من أغذية الحيوانات المستعملة في الأردن وهي الذرة والصويا ومركز الدجاج ، خلطة الدجاج المطحونة ، خلطة الدجاج الخشنة ، خلطة طعام البقر ، الشعير ، النخالة ، الفصة (البرسيم المصرى) والعدس. جمعت هذه العينات في الفترة ما بين شباط ١٩٨٠ وآذار ١٩٨١ من معظم مناطق الضفة الشرقية.

لقد أجريت الفحوص اللازمة لهذه العينات للتحقق من وجود سموم الافلاتوكسن Aflatoxins وسم الاستريجماتوسيستن Sterigmatocystin الفطرية. كذلك أجريت الفحوص المناسبة للكشف عن الفطريات التي تفرز سموم الافلاتوكسن. من خلال الدراسة وجد الافلاتوكسن B_1 في عينة واحدة من الذرة بتركيز يقارب ٥٣ جزء في البليون وكذلك الافلاتوكسن B_2 بتركيز يقارب ١٣ جزء في البليون بينما لم يتبين وجود الاسترجماتوسيستن في أى من العينات المفحوصة لهذا الغرض.

لقد بحث عن الفطريات التي يشتبه أن لها القدرة على افراز سموم الافلاتوكسن باستخدام الفحوص المجهرية والمورفولوجية وتم عزل ٢٣٨ عزلة من مجموعة أسبرجلس فلافس *Aspergillus flavus* المعروفة بافرازها لهذه السموم. وبعد التأكد من العزلات تبين أن معظمها من نوع أسبرجلس فلافس لنك اكس فرايز *A flavus* link ex fries ، والقليل منها من نوع أسبرجلس بارازيتكس سبير *A parasiticus* Spear ، أسبرجلس اوريزي *A oryzoe* وكذلك أسبرجلس تماري *A tamarii* . كذلك أختبرت صلاحية

بعض المواد الغذائية لتنمية الفطر وافراز السموم عليها وتبين أن مركز الدجاج ، وخلطة الدجاج والنخالة والعدس مواد تساعد على نمو الفطر وافراز السموم. وباستخدام الفصل الكروماتوغرافي بالصفائح الرقيقة وامتصاص الأشعة فوق البنفسجية بجهاز مقياس الطيف الضوئي والفحوص الكيماوية لتعريف السموم المفرزة تبين أن ما تفرزه هذه العزلات بصورة أساسية هي سموم أفلاتوكسن B_1 و B_2 والقليل منها له القدرة على افراز B_1 ، B_2 ، G_1 .

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