CHAPTER 7.0 BIOCHEMICAL STUDIES OF STARCH-BASED FOOD AFTER STORAGE AT DIFFERENT LEVELS OF WATER ACTIVITY

7.1 INTRODUCTION

The development of mycotoxin in stored agricultural produce is not well understood. For mycotoxin production to occur, fungal growth must have taken place. The filamentous form of fungi is adapted to growth over and through solid substrates by extension of the hyphal tip (Burnett and Trinci, 1979). The mycelium is able to breakdown the complex macromolecules of the substrate by the enzymes secreted and absorb the low molecular weight nutrients utilized for growth and metabolism. The hypha is a spatially polarised structure and, on a solid substrate, parts of the mycelium may reach the physiological status of the stationary phase, synthesising and excreting mycotoxins, while other parts of the same mycelium area actively growing and colonising fresh substrate (Moss, 1984).

Environmental conditions preventing fungal growth will also prevent mycotoxin formation. Conditions supporting toxin production are more restricted than those supporting growth (Lacey and Magan, 1991). Many studies have sought to determine how mycotoxin levels are affected by single factors (for example moisture, temperature or nature of substrate).

The most detailed accounts of the conditions and factors affecting growth and formation of aflatoxins by *A. flavus* are those of Austwick and Ayerst (1963), Hesseltine *et al.* (1966) and Jarvis (1971). These findings can be applied to other fungi, particularly of the Aspergilli group. The limiting temperature for the production of aflatoxin are reported as 12 - 41 °C with optimum production occurring between 25 - 32 °C (Lillehoj, 1983). Moisture requirements for growth of *A. flavus* and aflatoxin production in mature corn kernels shows that the fungus does not routinely exhibits extensive growth or toxin accumulation below

moisture contents in equilibrium with 85 % relative humidity (RH). However, at slightly higher moisture levels (86 -87 % RH), the fungus will grow rapidly and produce aflatoxin. Reports indicate that a significant amount of aflatoxin can be produced in mature corn kernels at 90 % RH within 48 hrs (Davis and Diener, 1983).

Many different mycotoxins have been isolated and characterised from fungi growing on stored agricultural commodity. However, the production of mycotoxins has been studied mostly at optimum A_W and temperatures on either rich laboratory media or autoclaved wheat, barley or rice. Very few studies have considered the effects of environmental factors, especially water activity on mycotoxin production by the normal microflora of food. During storage conditions of commodity, any number of fungal species may be growing and producing toxins. The predominance of any one species depends upon the interactions of unknown ecological factors which in turn influences the production of mycotoxins by a particular species. For example, *A. niger* has long been recognised as a potentially important competitor of *A. flavus* (Ashworth *et al.*, 1965; Joffe, 1969). Also, in laboratory experiments using autoclaved maize kernels, *A. flavus* produced no aflatoxin in the presence of *A. niger* or *Trichoderma viride*, while *T. viride* also prevented *A. flavus* sporulation (Wicklow *et al.*, 1980).

Most poisonings from consuming mouldy food probably are the result of combined effects of various mycotoxins, since rarely is a food product invaded by only one species of fungus, the mycotoxins are not produced in equal amounts and do not have equal toxic potency. From the toxicological standpoint, it is the

mycotoxin not the mold, which is important in the final analysis. The presence of a fungus does not necessarily indicate the presence of a specific mycotoxin, however, by determining the fungi present in a particular foodstuff and knowledge of the profile of mycotoxins and metabolites produced by each species (Frisvad, 1986; Svendsen and Frisvad; 1994; Andersen *et al.*, 1995) can reduce the number of mycotoxins needed to be analysed. Thus, identification of the toxicologically important metabolites of fungi invading food products is essential, since the toxicological evaluation of a product must rest solely on the presence as well as the amount of toxic metabolites required for making an assessment of the potential harm from any mycotoxin.

Chemical analysis of mycotoxins relied on extractions of the mycotoxin in a solvent that solubilized the toxin with a minimum of extraneous material, cleanup of the extract with a minimum loss of toxin, and finally, quantitation. Many workers have reported various analytical methods for determining mycotoxins produced by fungi growing on foods, most of which are used to measure only one toxin at a time. Since food are often contaminated with various fungi and that some fungi possess the ability to simultaneously produce more than one toxin (Richard and Gallagher, 1979), a multimycotoxin method of analysis is required. Several reports have appeared on multimycotoxin analysis (Stoloff *et al.*, 1971; Roberts and Patterson, 1975; Takeda *et al.*, 1979; Scott, 1995). It is virtually impossible to assess the relative scientific and practical merits of the different reports.

Yin (1969) had determined that acetonitrile containing a small proportion of water was a good selective solvent for aflatoxins, ochratoxin A and

zearalenone. Stoloff *et al.* (1971) also found that this solvent is also selective in regard to interfering background for the rest of the subject mycotoxin and potassium chloride prevents emulsion formation. Ammonium sulphate (30% aqueous solution) has been used to cleanup peanuts, rice, wheat, barley, cassava and corn samples (Scott *et al.*, 1981; Soares and Rodriguez-Amaya, 1985) and in mixed feed extracts (Romer *et al.*, 1978) for the determination of trichothecene and ochratoxin A.

Conventional analytical methods that have been developed over the past 25 years typically employ biological assay, thin-liquid chromatography, gas chromayography or mass spectrophotometry. High performance liquid chromatography (HPLC) has become the method of choice for mycotoxin analysis due to the advantages of good resolution, high degree of precision, reproducibility and sensitivity (Hsieh *et al.*, 1979; Pons, 1976). A systematic method for characterizing and quantitating mycotoxin is needed, and the method must be capable of determining the real state of fungal contamination in markets and fields. For the analysis of large numbers of samples, it is desirable to have available simple, rapid, economical and practical screening procedures.

The biological activity of mycotoxins are manifold which include acute and chronic toxicity, cytotoxicity, neurotoxicity, immunosuppresive activity, teratogenecity, mutagenecity, carcinogenicity, antitumour properties, insecticidal effects, antimicrobial properties and phytotoxicity (Betina, 1989).

The toxic effects of mycotoxins have been evaluated in various models i.e. chick embryo, day-old chick, ducklings, rabbit skin test and mouse water refusal (for trichothecenes), fish, mice, bull spermatozoa, amphibians and rats. In

vertebrate assays, besides being laborious and puts severe restrictions on the number of fungal isolates and culture conditions that can be tested, Cole (1984) has pointed out some disadvantages and problems of special test systems. For example, the chick embryo assay has essentially the same disadvantage as any injection-type bioassay in that it bypasses the normal digestion and absorption processes of the digestive tract and can be regarded as an artificial mean of administration. A major consideration concerning the validity of tests with intact vertebrate animals as primary mycotoxin screening system is in the mode of administration of the test material. The use of administration routes other than oral (i.e. intraperitoneal, intravenous and subcutaneous) may lead to erroneous conclusions concerning the toxicity of a fungal metabolite and, therefore, the toxigenic nature of a fungus (Panigrahi, 1993). Nevertheless, mycotoxin bioassay systems should be readily available, economical and simple to perform.

In spite of possible risks of false positive or false negative results, non-vertebrates are frequently used in mycotoxicology. They include brine shrimp, insects, mollusc eggs, planaria or nematodes. It must be borne in mind, however, that the screening system used may not be sensitive to some fungal metabolites that are toxic to higher animals.

Bioassays for the detection of toxic metabolites of fungi using the larvae of *Artemia salina L.*, commonly known as brine shrimps, have been extensively evaluated by several authors (Brown *et al.*, 1968; Harwig and Scott, 1971; Eppley, 1974; Scott *et al.*, 1980; Schmidt, 1985; 1989; Panigrahi and Dallin, 1994). Also, the organism has been widely used, examples are for the quantitation of the potency of anaesthetics (Robinson *et al.*, 1965), evaluation of toxicity of

dichloro-diphenyl-trichloroethane (Grosh, 1967) and other insecticides (Michael *et al.*, 1956), of heavy metals (Chanh and Mamy, 1963) and antibiotics (Delcambe, 1955), and for testing the inhibitory effects of carcinogens on the hatching of the eggs (Buu-hoi and Chanh, 1970).

A. salina is a primitive crustacean found in salt lakes in all continents but not in the sea. The features which make the brine shrimps valuable as test organism have been characterized by Schmidt (1989). They exhibits high sensitivity against a broad range of compounds and the viability of the eggs even under adverse conditions. Maintaining cultures of the organisms becomes unnecessary as hatching can be obtained within 24 to 30 hrs. The environment of the larvae from hatch until end of the experiment can be easily controlled. The eggs can be purchased from pet shops because the shrimps are widely used as food for tropical fish.

Hence, this study is concern with the detection and quantitation of mycotoxins formed by the natural microflora of six starch-based food upon storage at different A_W. For the analysis of mycotoxins, the extraction and cleanup method adapted from Takeda *et al.* (1979) and Soares and Rodriguez-Amaya (1985) and quantitation by using reversed-phase HPLC modified from Frisvad (1987) was evaluated. It is also aimed to develop an HPLC method that resolves and detects nanogram amounts of seven types of mycotoxins either as individual standards or in a mixture of standards.

The starch-based food crude extracts were also tested for toxicity against brine shrimp to support the HPLC analysis for the possible presence of toxic compounds other than those being analysed. The brine shrimp is a suitable

screening system for testing toxicity from metabolites of fungi and due to the limited volume of sample extracts available, this test was adopted because it can be conducted in a small volume (less than 0.5 mls), thus requiring a small quantity of the toxin.

7.2 MATERIALS AND METHODS

7.2.1 Extraction and reversed phase HPLC methods for detection and quantitation of mycotoxins

7.2.1.1 Apparatus

Equipment specified is not restrictive; other suitable equipment can be substituted.

i. Apparatus for sample size reduction - Braun grinder.

ii. Liquid chromatographic equipment - Bio-Rad High Resolution Liquid Chromatography (HRLC) gradient systems having

a) a series 800 HRLC gradient module, consisting of two series 1350 soft-start pumps, a model 7125 injector with a 20 µl loop and an HRLC system interface,

b) Series 800 HRLC data station, consisting of an IBM-compatible computer and HRLC software,

c) Acer Laser III printer and

d) Model 1706 UV/VIS detector.

iii. HPLC column - 250 mm x 4.6 mm i.d., 10 µm Econosil C_{18} (Alltech).

iv. Rotary evaporator.

v. Glasswares - funnel, 150 mm with fluted Whatman No. 1 filter paper to fit, 250-ml separatory funnel, 600-ml beaker, 200-ml measuring cylinder and

12-ml glass vials with PTFE lined screw caps. All glasswares exposed to mycotoxins were washed as described in Appendix B.

7.2.1.2 Reagents

All chemicals and reagents used are analytical grades unless indicated otherwise.

i. Extraction solvents - acetonitrile (HPLC grade), potassium chloride, 4% 40.0 gl^{-1}); sulphuric acid, 20 %; chloroform (HPLC grade).

ii. Clarifying agent - ammonium sulphate, 30 % (300.0 gl^{-1}).

iii. Diatomaceous earth.

iv. HPLC elution solvent - acetonitrile (HPLC grade), glass-distilled deionised water, trifluoroacetic acid, 0.05% (Uvasol, for spectroscopy, E. Merck).

v. Mycotoxin standards - patulin, ochratoxin A and cytochalasin E were dissolved in chloroform while aflatoxin B_1, aflatoxin G_1, sterigmatocystin, and griseofulvin were dissolved in chloroform-methanol (1+1) to obtain a stock solution of 5 $mgml^{-1}$.

Mycotoxin standards were purchased from Sigma Chemicals.

7.2.1.3 Sample preparation

Grain samples i.e. rice and glutinous rice were ground to 20 mesh in a grinder. All ground samples were thoroughly mixed with an aluminium spatula before analytical samples were taken.

7.2.1.4 Extraction and cleanup

20 g powdered sample was weighed into a 250-ml flask with stopper, 200 ml of 20% H_2SO_4 - 4% KCL - acetonitrile (2+20+178) was added. The flask was stoppered and shaken for 30 min on a rotary shaker at 250 rpm. The extract was filtered through fluted Whatman No. 1 filter paper and 150 ml aliquots of filtrate was transferred to a 600-ml beaker.

150 ml clarifying solution, ammonium sulphate (30%) and 3 g of diatomaceous earth was added to this aliquot and stirred with a glass rod. The extract was filtered through fluted Whatman No. 1 filter paper and 100 ml of the upper layer (acetonitrile) was transferred into a 250-ml separatory funnel. 50 ml water was added and the aqueous acetonitrile layer was extracted with 50 ml chloroform by shaking for 5 mins. Extraction was repeated with 30 ml and then 10 ml chloroform.

The extracts were combined and evaporated to dryness in vacuo at 45 °C on a rotary evaporator. The residue was dissolved in a known volume of chloroform and transferred into glass vials, weighed and stored in a freezer (-18 °C) before analysis.

7.2.1.5 Recovery and reproducibility studies

The proposed quantitative method was evaluated by using spiked samples. Autoclaved samples of each type of starch-based food were spiked to levels of 15, 50 and 75 μgkg^{-1} of patulin and griseofulvin, 50, 100 and 150 μgkg^{-1} of aflatoxin G_1, aflatoxin B_1 and sterigmatocystin, 75, 100 and 150 μgkg^{-1} of ochratoxin A

and 2500, 2800 and 3000 μgkg^{-1} of cytochalasin E by weighing a 20 g sample in a beaker and adding the desired amount of mycotoxin mix with a pipette.

Mycotoxin mix was prepared by taking appropriate aliquots of each mycotoxin from the stock (5 $mgml^{-1}$) and mixed. The mixtures were then evaporated under nitrogen and dissolved in the requisite amount of chloroform.

The mixed standard solutions were allowed to drop at different points on the center top of the mass. No mixing was done, to prevent standard solution from adhering to the walls of the container. The solvent from the standard solution was allowed to dry naturally overnight. The spiked samples were extracted as above (7.2.1.4). The entire procedure was repeated 3 times for each level of spiking for each starch-based food.

7.2.1.6 Mycotoxin standard solutions

10 µl aliquots of patulin and griseofulvin were taken from the stock (5 $mgml^{-1}$) and diluted with 490 ul chloroform to obtain a working standard solution of 0.1 $mgml^{-1}$. Similarly, 10 µl of aflatoxin G_1 and sterigmatocystin, 20 µl of aflatoxin B_1 and ochratoxin A and 125 µl of cytochalasin E and were taken from the stock (5 $mgml^{-1}$) and diluted with appropriate amounts of chloroform to obtain a working standard solutions of 0.2 $mgml^{-1}$, 0.4 $mgml^{-1}$ and 2.5 $mgml^{-1}$ respectively.

Mycotoxin standard mixture was prepared by taking appropriate aliquots of each mycotoxin stock and mixed. The mixture were then evaporated under nitrogen and dissolved in requisite volume of chlorofom to provide a mycotoxin mix standards ranging from 0.1 - 2.5 mg of mycotoxins per ml. The mycotoxin

mix and single mycotoxins were suitably diluted to provide a range of concentration to obtain a standard curve.

Before storage, after aliquots have been removed, the vials containing the standards were weighed to the nearest mg and recorded for future reference. Vials were wraped tightly in aluminium foil and stored at 0 °C. When standards are to be used after storage, the vials were reweighed and any change recorded. To avoid incorporation of water by condensation, all standards were brought to room temperature before use. The aluminium foil was not removed from the vials until contents have reached room temperature.

7.2.1.7 HPLC analyses

The gradient solvent system used by Frisvad (1987) and Frisvad and Thrane (1987) was modified slightly. With water designated as solvent A and 0.05% trifluoroacetic acid in acetonitrile as solvent B, the solvent programme and the flow-rate used was as follows:

Time (mins)	solvent A %	solvent B %	Flow-rate ($ml min^{-1}$)
0	90	10	1.00
1.00	90	10	1.00
2.00	50	50	1.00
30.00	10	90	0.80
38.00	10	90	1.00
39.00	90	10	1.00
40.00	90	10	1.00

All mycotoxins were detected at 254 nm.

7.2.2 The production of mycotoxins in starch-based food stored at different levels of water activity

7.2.2.1 Incubation of starch-based food

The effect of storing six types of starch-based food at different levels of A_W on the production of mycotoxins were determined by analysing incubated samples from Chapter 4 (described in Method 4.2.1). At time intervals of 0, 7, 26, 54 and 96 days, 20 g samples were taken for mycotoxin analysis.

7.2.2.2 Analysis for mycotoxins

Simultaneous extraction of seven types of mycotoxins were done as above (7.2.1.4). The extracts were then analysed for the presence of patulin, aflatoxin G_1, aflatoxin B_1, griseofulvin, cytochalasin E, ochratoxin A and sterigmatocystin by reversed-phase HPLC method described above (7.2.1.7). Quantitation were done on the basis of the standard curves obtained in Appendix B based on the peak height response.

7.2.3 Toxicity of starch-based food extracts on the brine shrimp, *Artemia salina L*

7.2.3.1 Breeding of the larvae

In this experiment, artificial salt water (Marinemix) of specific gravity 1.01 - 1.02 was used. Its pH was adjusted to 8.0 with sodium hydroxide (1M). A pinch of brine shrimp eggs (originated from the Great Salt Lake) was added to 100 mls of the medium in a 250-ml beaker temperated to 28 °C in the light and intensively

aerated. Larvae emerged from eggs after 18 - 24 hrs. After hatching, light was eliminated.

Brine shrimp of uniform vitality was obtained by selecting larvae that swim from a darkened to an illuminated environment (Robinson *et al.*, 1965 and Schmidt, 1989). The brine shrimp eggs are placed on one side of a barrier in a rearing dish. The hatched larvae are able to swim through the openings in the barrier. A light source at the side of the dish attracts the swimming larvae through the barrier and also causes the larvae to congregate in one location where sufficient numbers of them may easily be drawn up into a pipette.

7.2.3.2 Method of testing

100 µl of chloroform extracts of six starch-based foods exposed to levels of A_W ranging from 0.65 - 0.98 were pipetted and allowed to evaporate completely at 45 °C. When cooled, dimethyl sulphoxide (DMSO) was added to obtain a stock solution of concentration ranging from 100 -200 $mgml^{-1}$ extract.

The concentrations were increased or reduced, if necessary to get at least four different concentrations required for calculation of LC_{50}.

Volumes of 25 µl of extract in DMSO were pipetted into 1.5 cm diameter x 1.5 cm deep plastic wells and larval suspension was added to make up to 1.5 mls. Hence, for a complete test with each extract, an average of 10 - 20 larvae were treated per dose in triplicate for each of the four concentrations. Simultaneously, two controls were carried out i.e. 25 µl DMSO and 25 µl of each starch-based food extracts at Day 0, having the same range of concentration as the test extracts.

The wells were checked at zero time by a stereoscopic microscope at a magnification of 15 x 0.66 for a possible presence of dead larvae. The wells were covered to prevent evaporation of the solutions. The extract were allowed to act for 18 hrs at 20 - 22 °C. After 18 hrs dead larvae were counted in each well by means of a stereoscopic microscope. Then the remaining living larvae were killed with a drop of formalin and the total number of larvae was counted.

7.2.3.3 Analysis of bioassay data

Mortality (including moribund shrimp) was corrected for both control using Abbott's formula (1925):

$$\text{Corrected mortality (\%)} = \frac{\text{Treated mortality - Control mortality at Day 0}}{\text{100 - Control mortality at Day 0}} \times 100$$

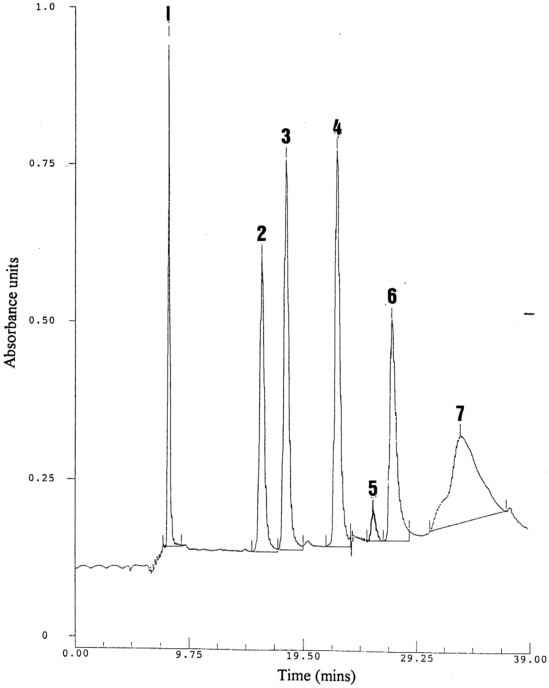
The bioassay data were analysed by the Probit method of Finney (1971) to obtain LC_{50} values.

7.3 RESULTS

7.3.1 Resolution of seven mycotoxins by reversed-phase HPLC

The chromatogram shown in Fig. 7.1 illustrates the resolution of a mixture of seven mycotoxins with the HPLC system used. The seven mycotoxins were completed in 34 mins at the flow-rate used. Seven injections of individual and mixed mycotoxin standards over a 5-day period gave retention times as shown in Appendix D(7.1) and summarized in Table 7.1.

Fig. 7.1 HPLC of a mixture of seven mycotoxins



Seven mycotoxins eluted from 10 μm Econosil (C_{18}) reversed-phase column using water and 0.05% trifluoroacetic acid in acetonitrile and detected by UV-Vis detector at a wavelength of 254 nm.

Peak 1 : 0.1 $mgml^{-1}$ Patulin
Peak 2 : 0.2 $mgml^{-1}$ Aflatoxin G_1
Peak 3 : 0.4 $mgml^{-1}$ Aflatoxin B_1
Peak 4 : 0.1 $mgml^{-1}$ Griseofulvin
Peak 5 : 1.575 $mgml^{-1}$ Cytochalasin E
Peak 6 : 0.4 $mgml^{-1}$ Ochratoxin A
Peak 7 : 0.15 $mgml^{-1}$ Sterigmatocystin

The mycotoxins purchased were used without additional purification. There were no contaminants observed by HPLC of the individual mycotoxin standards. Baseline resolution was obtained between all mycotoxins and all the peaks were sharp except for sterigmatocystin which gave a broad peak (Fig. 7.1).

The relationship between peak heights converted to absorbance and amount of mycotoxins injected was linear for each mycotoxin over the concentration range studied. The data and standard curves obtained for each mycotoxin were displayed in Appendix B.

Table 7.1 Reproducibility of retention times for seven mycotoxins by HPLC with coefficient of variation

Mycotoxins	Statistics*				
	Retention time (sec)		Mean retention (min)	Std. deviation (sec)	Coeff of variation (%)
	Range	Mean			
Patulin	479.40 - 484.80	482.40	8.04	2.20	0.40
Aflatoxin G_1	958.80 - 966.60	963.60	16.06	3.60	0.40
Aflatoxin B_1	1079.40 - 1087.20	1083.60	18.06	3.40	0.30
Griseofulvin	1329.60 - 1342.20	1339.20	22.32	6.50	0.50
Cytochalasin E	1530.00 - 1543.80	1537.20	25.62	4.40	0.30
Ochratoxin A	1615.20 - 1639.20	1627.80	27.13	8.70	0.50
Sterigmatocystin	1975.20 - 2013.00	1990.80	33.18	14.30	0.70

* Based on successive injections of 20 µl aliquots of individual and mixed mycotoxin standards, n = 7.

Precision was evaluated by injecting seven 20 µl aliquots of mixed mycotoxin standards having a concentration ranging from 0.1 - 1.8 $mgml^{-1}$ over a 2-day period. Reproducibility of the peak height measurements was good as shown in Appendix D(7.2), with coefficients of variation of 1.5 - 11.0 %, representing the combined errors of HPLC resolution, injection and detection as summarized in Table 7.2.

Table 7.2 Peak height reproducibility in the separation of seven mycotoxins by HPLC

Mycotoxins	Statistics *			
	Peak height (mm)		Std. deviation (mm)	Coeff. of variation (%)
	Range	Mean		
Patulin	181.0 - 190.0	185.7	3.2	1.7
Aflatoxin G_1	109.0 - 119.0	113.6	4.2	3.7
Aflatoxin B_1	133.0 - 143.0	138.3	3.4	2.4
Griseofulvin	145.0 - 150.0	147.0	2.2	1.5
Cytochalasin E	10.0 - 14.0	11.8	1.3	11.0
Ochratoxin A	81.0 - 89.0	84.3	3.4	4.0
Sterigmaocystin	35.0 - 40.0	37.1	1.8	4.8

* Based on successive 20 µl injections of mixed mycotoxin standards at detector range of 0.32, n = 7

Detection limits and sensitivity for the mycotoxins are listed in Table 7.3. The criterion for determining the detection limits was simply the lowest concentration at which a mycotoxin would produce a small peak (2% of full scale) on the recorder when the detector was set at a range of 0.32.

Among the mycotoxins screened, the detection limits and peak ratios indicate that patulin is most sensitive, being detected at a minimum concentration of 31 ng followed by griseofulvin at 42 ng, aflatoxin G_1 at 125 ng, sterigmatocystin at 188 ng, aflatoxin B_1 at 200 ng and ochratoxin A at 250 ng. Cytochalasin E is least sensitive being detected at a minimum concentration 11.25 µg.

Table 7.3 Detection limit and sensitivity of mycotoxins by HPLC

Mycotoxins	Min. peak height (mm)	Detection limits * (ng)	Sensitivity (mm peak height / ng *)	Peak ratios
Patulin	5.6	31	0.1800	409.1
Aflatoxin G_1	4.0	125	0.0300	72.7
Aflatoxin B_1	4.4	200	0.0200	50.0
Griseofulvin	4.0	42	0.0900	218.2
Cytochalasin E	5.0	11250	0.0004	1.0
Ochratoxin A	3.5	250	0.0140	31.8
Sterigmatocystin	4.5	188	0.0240	54.5

* Calculated to maximum sensitivity at detector range of 0.32.

7.3.2 Recovery and reproducibility studies

An evaluation of the extraction method was conducted on spiked samples. Autoclaved ground samples of rice, glutinous rice, riceflour, glutinous riceflour, wheatflour and cornflour were spiked to 3 levels of each mycotoxins. Results obtained from triplicate determinations at each levels are shown in Table 7.4. Recoveries were also evaluated at the detection limits for each mycotoxin.

Percentage recoveries and range of coefficients of variation in six starch-based food were > 90% and 0.6 - 9.4% for patulin respectively, > 91% and 0.4 - 6.9% for aflatoxin G_1 respectively, > 92% and 0.3 - 7.4% for aflatoxin B_1 respectively, > 83% and 1.1 - 18% for griseofulvin respectively, > 89% and 0.6 - 3.5% for cytochalasin E respectively, > 88% and 0.8 - 15% for ochratoxin A respectively, and > 90% and 1.0 - 11.1% for sterigmatocystin respectively.

The quantitation limits were higher for patulin in rice, for aflatoxin G_1 in wheatflour, for aflatoxin B_1 in glutinous rice, for griseofulvin in glutinous riceflour, for cytochalasin E in wheatflour, for ochratoxin A in wheatflour and for sterigmatocystin in cornflour (Table 7.4).

Table 7.4 Recovery and reproducibility data for mycotoxins in spiked starch-based food

Starch-based foods		Rice		Glutinous rice		Riceflour		Glutinous riceflour		Wheatflour		Cornflour	
Mycotoxins	Amount added (μgkg^{-1})	Av. rec. %	CV %	Av. rec. %	CV %	Av. rec. %	CV %	Av. rec. %	CV %	Av. rec. %	CV %	Av. rec. %	CV %
Patulin	15	90	9.3	93	5.4	97	9.4	97	3.4	93	7.4	93	7.1
	50	100	0.6	100	5.6	98	6.2	100	6.2	95	5.3	95	5.3
	75	100	1.4	100	1.0	99	3.5	97	3.5	94	1.2	94	0.7
Aflatoxin G_1	50	96	1.0	95	6.9	95	4.4	93	3.3	@	@	94	5.4
	100	98	1.1	97	2.8	98	2.6	92	2.7	92	2.7	96	1.6
	150	100	2.2	98	1.7	99	1.9	97	3.4	91	0.9	93	2.0
Aflatoxin B_1	50	95	3.8	93	6.2	94	3.3	95	5.3	95	5.3	98	2.1
	100	98	1.6	98	2.6	94	1.9	96	1.1	95	0.3	100	5.0
	150	97	3.4	100	7.4	96	4.0	95	1.9	96	2.1	92	1.8
Griseofulvin	15	97	3.4	97	3.4	93	9.0	83	18.0	90	11.3	97	3.4
	50	100	2.9	100	10.0	94	5.3	95	5.3	91	1.1	93	3.2
	75	100	4.0	92	5.2	96	2.1	97	3.5	90	3.7	95	2.0
Cytochalasin E	2500	@	@	@	@	@	@	@	@	@	@	@	@
	2800	95	1.9	95	0.8	92	1.1	92	1.0	89	1.1	90	1.0
	3000	96	3.5	97	0.8	97	1.7	96	1.7	92	0.6	91	1.9
Ochratoxin A	75	100	6.0	97	3.5	96	3.7	90	3.7	88	3.1	94	0.8
	100	100	15.0	98	2.6	95	1.3	98	2.7	92	2.7	93	3.0
	150	92	9.1	98	1.7	100	1.1	95	1.8	91	1.6	92	1.8
Sterigmatocystin	50	90	11.1	95	5.3	96	5.5	98	2.6	93	4.5	@	@
	100	96	3.0	98	2.7	98	2.4	98	2.6	96	1.6	96	1.3
	150	96	5.3	100	3.3	98	2.0	97	2.8	97	1.0	93	9.4

* Based on 3 determinations for each level **Key :-** @ inappropriate for quantitation; Ave rec.: average recovery; CF : coefficient of variation

7.3.3 Quantitation of mycotoxins in starch-based food stored at different levels of water activity

The composite samples of each starch-based food obtained from the retail outlets (day 0) did not indicate the presence of mycotoxins screened. Five replicate samples were extracted and analysed. The chromatogram displayed in Fig. 7.2 (a and b) of each food did not show any peak matching with any seven mycotoxin standards.

Chromatograms showing the presence of mycotoxins in starch-based food stored at different levels of A_W and storage period are given in Appendix D(7.3). The types and concentration of mycotoxins detected in each starch-based food after day 0 are summarized in Table 7.5. None of the toxins screened were detected at 0.65 and 0.75 A_W even after 96 days of storage.

Upon screening for mycotoxins in ordinary rice grains which were stored at different levels of A_W for 96 days, six types of mycotoxins were detected. Patulin was detected at 96 days when stored at 0.95 A_w and 54 days at 0.98 A_w at concentrations of 0.025 $\mu gg^{-1}$and 0.126 μgg^{-1} respectively. The incidence of aflatoxin B_1 occurred at A_W ranging from 0.80 - 0.90 after 26 days of storage with minimum and maximum concentrations of 0.120 and 0.267 μgg^{-1} at 0.80 A_w, 0.130 and 0.162 μgg^{-1} at 0.85 A_w and 0.063 and 0.082 μgg^{-1} at 0.90 A_w. At 0.95 0.267 μgg^{-1} aflatoxin B_1 was detected at 54 days. Hence, aflatoxin B_1 occurred most regularly in ordinary rice grains, the highest level being detected at 0.80 and 0.95 A_w. Aflatoxin G_1 and griseofulvin were detected at water activities of 0.95 and 0.98 only. At 0.95 A_w, 0.412 μgg^{-1} aflatoxin G_1 was detected at 26 days while at 0.98 A_w 0.268 and 0.124 μgg^{-1} were detected at 54 and 96 days

Fig.7.2a HPLC of starch-based food at day 0

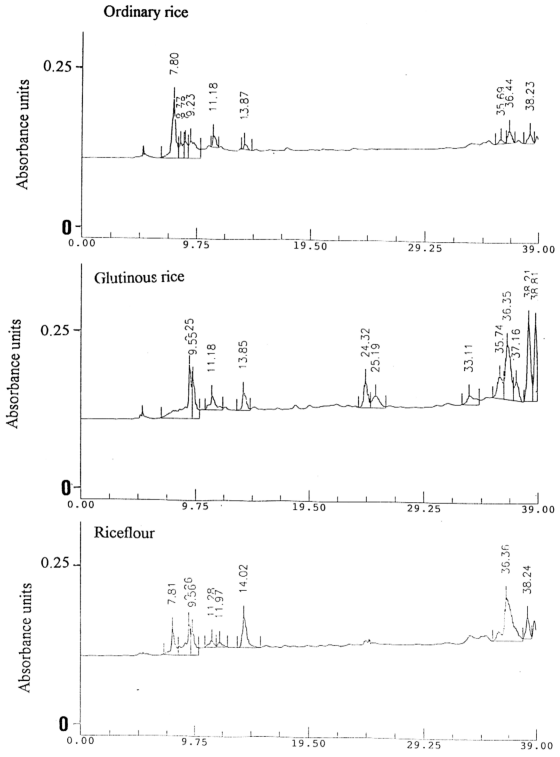


Fig. 7.2b HPLC of starch-based food at day 0

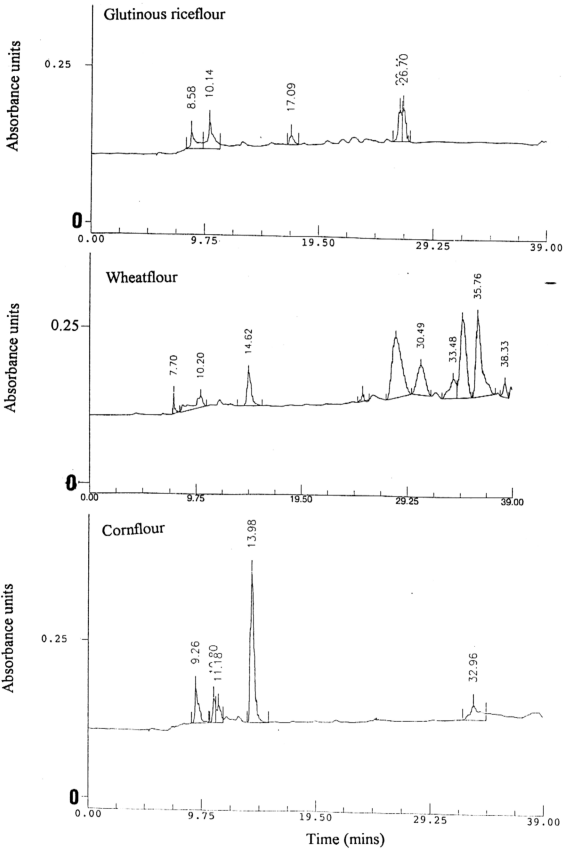


Table 7.5 The incidence of mycotoxins ($\mu g g^{-1}$) in starch-based food stored at different levels of A_w at 25 °C

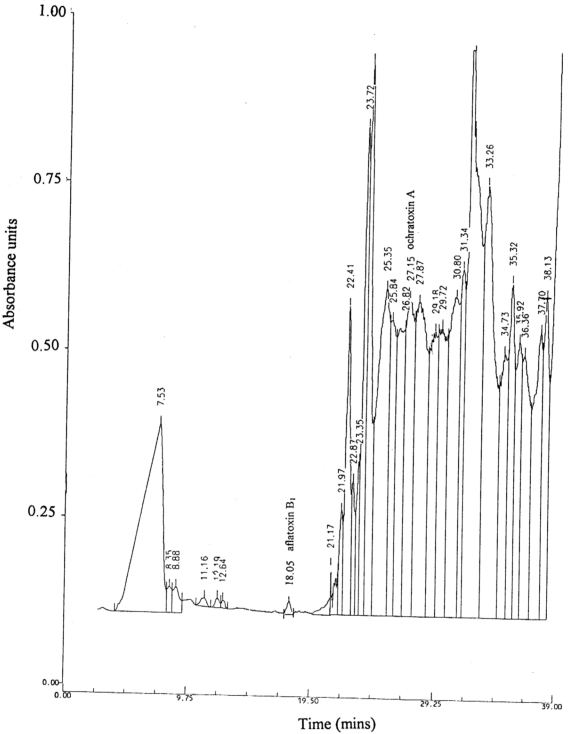
Water activity	Storage time (days)	Ordinary rice						Glutinous rice				Riceflour			GRF	Wheatflour				CF
		Pat	B_1	G_1	GSF	ST	OA	Pat	B_1	G_1	OA	Pat	B_1	GSF	B_1	B_1	G_1	GSF	ST	B_1
0.65	7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	26	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	54	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	96	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.75	7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	26	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	54	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	96	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.80	7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	26	-	0.267	-	-	-	-	-	-	-	5.510	-	0.178	-	-	-	-	-	-	-
	54	-	0.120	-	-	0.060	-	-	-	-	6.202	-	0.242	-	-	0.140	0.062	-	-	-
	96	-	0.150	-	-	0.062	-	-	-	-	6.112	-	0.640	-	0.057	0.070	0.050	-	-	-
0.85	7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	26	-	0.130	-	-	0.070	-	-	-	-	7.656	-	0.085	-	-	0.210	0.080	-	-	-
	54	-	0.143	-	-	0.083	-	-	-	-	8.207	-	0.059	-	0.265	0.200	0.090	-	0.050	-
	96	-	0.162	-	-	0.071	-	-	-	-	4.919	-	0.481	-	0.179	0.140	0.070	-	0.087	-
0.90	7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	26	-	0.063	-	-	0.050	-	-	-	-	3.182	-	0.642	-	0.800	0.078	-	-	-	0.059
	54	-	0.082	-	-	0.180	-	0.026	0.929	-	-	-	0.839	-	0.220	0.140	-	0.107	0.180	0.070
	96	-	0.071	-	-	-	10.250	0.051	0.981	-	-	-	0.823	-	0.110	0.180	-	0.098	0.140	0.069
0.95	7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	26	-	-	0.412	-	0.480	-	-	-	-	8.878	-	0.823	0.031	0.890	-	-	0.156	-	-
	54	-	0.267	-	-	0.360	-	-	4.742	4.791	-	0.351	0.713	0.352	0.980	-	-	0.315	0.702	-
	96	0.025	-	-	0.224	-	-	-	-	-	8.169	0.028	0.546	0.022	0.960	-	-	0.184	0.612	-
0.98	7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	26	-	-	-	0.272	-	-	-	-	3.168	-	-	-	-	-	-	-	-	-	-
	54	0.126	-	0.268	-	-	-	-	-	4.696	-	-	0.658	0.183	0.080	-	-	-	-	-
	96	-	-	0.124	-	-	-	0.052	-	0.189	-	0.062	0.520	0.128	0.140	-	-	-	-	-

Note:- Pat : Patulin; B_1 : Aflatoxin B_1; G_1: Aflatoxin G_1; GSF : Griseofulvin; ST : Sterigmatocystin; OA : Ochratoxin A
GRF : Glutinous riceflour; CF : Cornflour; - : not detected

respectively. Griseofulvin was detected at 96 days when stored at 0.95 A_w and at 26 days when stored at 0.98 A_w at concentrations of 0.224 μgg^{-1} and 0.272 μgg^{-1} respectively. The incidence of sterigmatocystin was also frequent at A_w between 0.80 - 0.95 after 26 days of storage. The minimum and maximum concentrations were 0.060 and 0.062 μgg^{-1} at 0.80 A_w, 0.070 and 0.083 μgg^{-1} at 0.85 A_w, 0.050 and 0.180 μgg^{-1} at 0.90 A_w and 0.360 and 0.480 μgg^{-1} at 0.95 A_w. Hence, the highest level of sterigmatocystin in ordinary rice was detected at 0.95 A_w. Ochratoxin A was detected only at 0.90 A_w after 96 days of storage at a high concentration of 10.250 μgg^{-1} (Fig. 7.3).

Four types of mycotoxins (patulin, aflatoxin B_1, aflatoxin G_1 and ochratoxin A) were detected when glutinous rice grains was stored at water activities ranging from 0.80 - 0.98. Concentrations of patulin detected at 54 and 96 days when stored at 0.90 A_w were 0.026 and 0.051 μgg^{-1} respectively while at 96 days stored at 0.98 A_w, it was present at a concentration of 0.052 μgg^{-1}. Aflatoxin B_1 was detected at 54 and 96 days when stored at 0.90 A_w at concentrations of 0.929 and 0.981 μgg^{-1}. At 0.95 A_w, 4.742 μgg^{-1} aflatoxin B_1 was detected at 96 days. Aflatoxin G_1 was present at concentration of 4.791 μgg^{-1} at 54 days when stored at 0.95 A_w. Concentrations of 3.168, 4.696 and 0.189 μgg^{-1} aflatoxin G_1 were detected at 26, 54 and 96 days respectively when stored at 0.98 A_w. Ochratoxin A was detected in glutinous rice grains stored at A_w ranging from 0.80 - 0.95 after 26 days of storage. The minimum and maximum concentrations detected at 0.80 A_w was 5.510 and 6.202 μgg^{-1}, at 0.85 A_w was 4.919 and 8.207 μgg^{-1} and at 0.95 A_w was 8.169 and 8.878 μgg^{-1}. At 0.90 A_w, 3.182 μgg^{-1} ochratoxin A was detected only at 26 days of storage. Hence,

Fig. 7.3 HPLC of ordinary rice grains stored at 0.90 A_w at 25 °C for 96 days



Aflatoxin B_1 was detected at a concentration of 0.071 μgg^{-1} at a retention time of 18.05 mins. Ochratoxin A was detected at a concentration of 10.250 μgg^{-1} at a retention time of 27.15 mins.

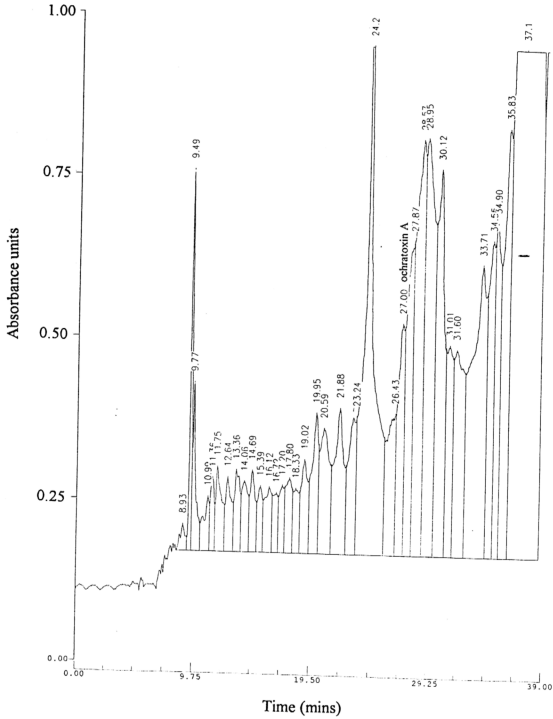
ochratoxin A was most frequently detected in glutinous rice grains, the highest concentration being detected at 0.95 A_w at 26 days (Fig. 7.4).

Three types of mycotoxins (patulin, aflatoxin B_1 and griseofulvin) were detected in riceflour stored at different levels of A_W. Patulin was produced at 0.95 A_W at levels of 0.351 and 0.028 μgg^{-1} at 26 and 54 days respectively and 0.062 μgg^{-1} occurred at 96 days when stored at 0.98 A_w . Aflatoxin B_1 was detected at $A_w > 0.80$ with minimum and maximum concentration of 0.178 and 0.640 μgg^{-1} at 0.80 A_w , 0.059 and 0.481 μgg^{-1} at 0.85 A_w , 0.642 and 0.839 μgg^{-1} at 0.90 A_w, 0.546 and 0.823 μgg^{-1} at 0.95 A_w and 0.520 and 0.658 μgg^{-1} at 0.98 A_w. Hence, aflatoxin B_1 was most frequent in riceflour, the highest level being detected at 0.90 A_w at 54 days (Fig. 7.5). Griseofulvin was present at 0.95 and 0.98 A_w with minimum and maximum concentrations of 0.022 and 0.352 μgg^{-1} at 0.95 A_w and 0.128 and 0.183 μgg^{-1} at 0.98 A_w.

When glutinous riceflour was stored at different levels of A_w for 96 days, only aflatoxin B_1 was detected at $A_w > 0.80$. At 0.8 A_w, 0.057 μgg^{-1} was detected at 96 days. The minimum and maximum concentrations at water activities of 0.85 was 0.179 and 0.265 μgg^{-1}, at 0.90 A_w was 0.110 and 0.800 μgg^{-1}, at 0.95 A_w was 0.890 and 0.980 μgg^{-1} and at 0.98 A_w was 0.140 and 0.080 μgg^{-1}. Hence, the highest level of aflatoxin B_1 in glutinous riceflour occurred at 0.95 A_w at 54 days (Fig. 7.6).

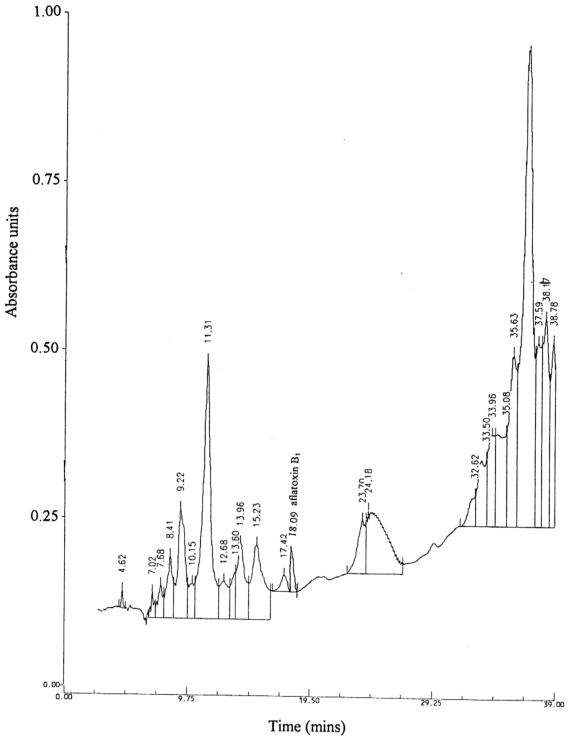
Four types of mycotoxins were detected in wheatflour when stored at different levels of A_W i.e. aflatoxin B_1, aflatoxin G_1, griseofulvin and sterigmatocystin. Aflatoxin B_1 was detected at water activities of 0.80, 0.85 and 0.90 and the minimum and maximum concentrations were 0.070 and 0.140 μgg^{-1},

Fig. 7.4 HPLC of glutinous rice grains stored at 0.95 A_w at 25 °C for 26 days



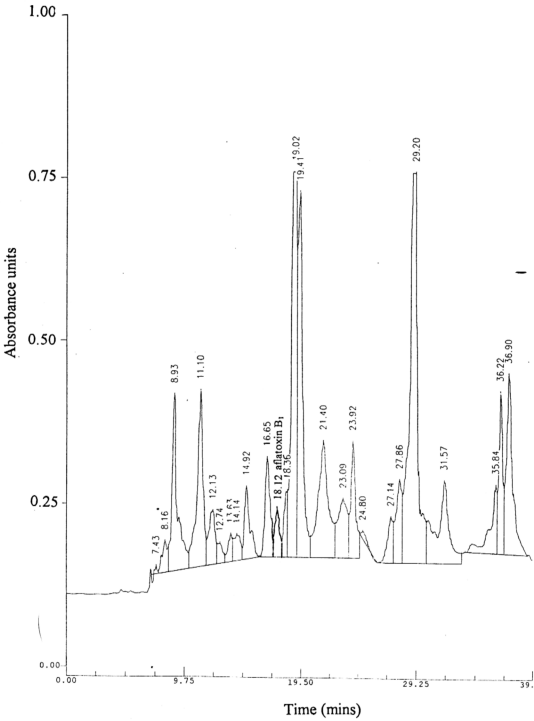
Ochratoxin A was detected at a concentration of 8.878 μgg^{-1} at a retention time of 27.00 mins.

Fig. 7.5 HPLC of riceflour stored at 0.90 A_w at 25 °C for 54 days



Aflatoxin B_1 was detected at a concentration of 0.839 $\mu g g^{-1}$ at a retention time of 18.09 mins.

Fig. 7.6 HPLC of glutinous riceflour stored at 0.95 A_w at 25 °C for 54 days



Aflatoxin B_1 was detected at a concentration of 0.980 $\mu g g^{-1}$ at a retention time of 18.12 mins.

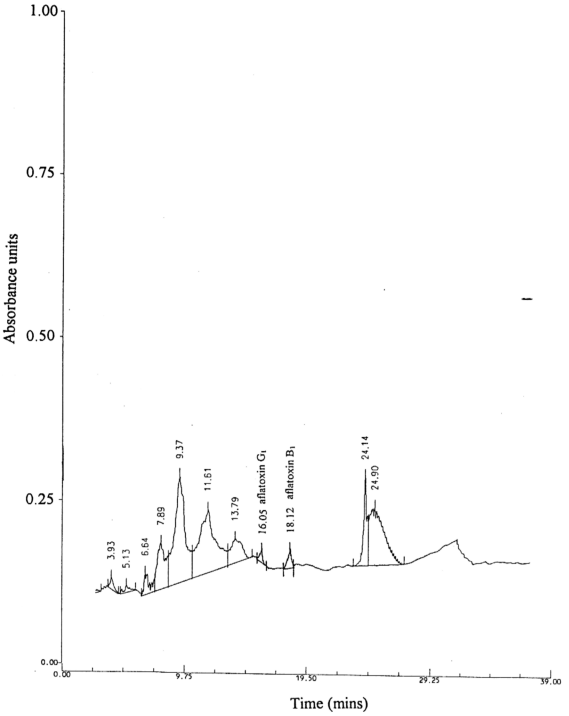
0.140 and 0.210 μgg^{-1} and 0.078 and 0.180 μgg^{-1} respectively. Aflatoxin B_1 was most frequent in wheatflour and the highest level was detected at 0.85 A_w at 26 days (Fig. 7.7). Aflatoxin G_1 was detected at 0.80 and 0.85 water activities with minimum concentrations of 0.050 and 0.070 μgg^{-1} respectively. The maximum concentrations were 0.062 and 0.090 μgg^{-1} being detected at 54 days. Griseofulvin was detected at a higher level of water activity of 0.90 and 0.95 with minimum concentrations of 0.098 and 0.156 μgg^{-1} respectively and the maximum concentrations were 0.107 and 0.315 μgg^{-1} respectively. Sterigmatocystin occurred at water activities between 0.85 - 0.95 after 54 days of storage. At 0.85 A_w, 0.050 μgg^{-1} was detected at 54 days and 0.087 μgg^{-1} at 96 days of storage. At 0.90 A_w, 0.140 μgg^{-1} was detected at 54 days and 0.180 μgg^{-1} at 96 days of storage while at 0.95 A_w, 0.612 μgg^{-1} was detected at 54 days and 0.702 μgg^{-1} at 96 days of storage. Hence, highest level of sterigmatocystin in wheatflour occurred at 0.95 A_w.

Aflatoxin B_1 was the only mycotoxin detected in cornflour when stored at different levels of water activity. It occurred at 0.90 A_w with a minimum concentration of 0.059 μgg^{-1} at 26 days and a maximum concentration of 0.070 μgg^{-1} after 26 days of storage (Fig. 7.8).

7.3.4 Toxicity of starch-based food extracts on the brine shrimp, *Artemia salina L*

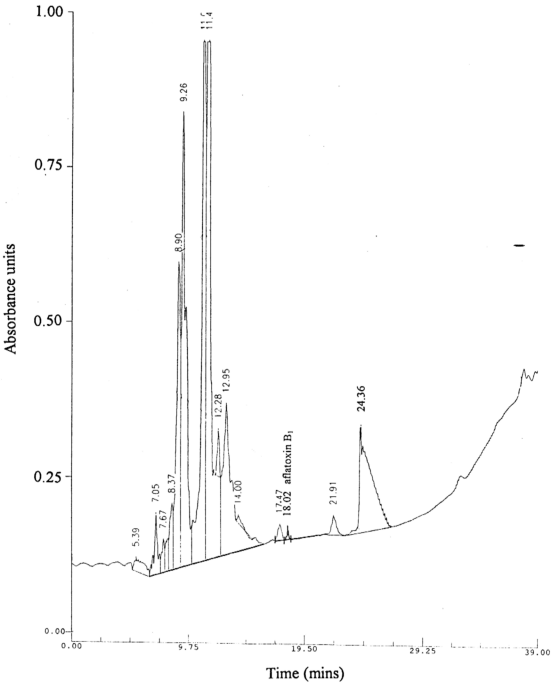
In this toxicity study, each starch-based food extracts were tested for biological activity on the brine shrimp, *A. salina* and their respective toxicity data are given in Tables 7.6 - 7.11. At the highest concentration tested, extracts which gave > 50% corrected mortality were analysed by the probit method of Finney

Fig. 7.7 HPLC of wheatflour stored at 0.85 A_w at 25 °C for 26 days



Aflatoxin B_1 was detected at a concentration of 0.210 $\mu g g^{-1}$ at a retention time of 18.12 mins. Aflatoxin G_1 was detected at a concentration of 0.080 $\mu g g^{-1}$ at a retention time of 16.05 mins.

Fig. 7.8 HPLC of cornflour stored at 0.90 A_w at 25 °C for 54 days



Aflatoxin B_1 was detected at a concentration of 0.070 $\mu g g^{-1}$ at a retention time of 18.02 mins.

(1971) to obtain LC_{50} value while extracts which gave < 50% corrected mortality were inappropriate for probit analysis.

The fifty percent lethal concentration or LC_{50} is commonly accepted as the basis for comparison in the investigation of relative toxicities among the compounds. The LC_{50} value is defined as the concentration that will kill half the population of the brine shrimp being tested when the toxicant is applied under constant experimental conditions.

Referring to Table 7.6, ordinary rice stored at water activities of > 0.80 gave > 50% mortality after a time period of 26 days and above. The LC_{50} of these extracts at these water activities and storage time were significant ($p = 0.05$). The lowest LC_{50} value of 257.2 μgml^{-1} and the highest LC_{50} value of 981.9 μgml^{-1} was exhibited by ordinary rice stored at 0.95 A_W for 96 days and at 0.80 A_W for 26 days respectively.

Referring to Table 7.7, storage of glutinous rice at 0.80 - 0.90 A_W shows more than 50% mortality after a time period of 26 days and above, while at 0.98 A_w at a time period of 7 days and above. The LC_{50} of the extracts were significant ($p= 0.05$). The lowest LC_{50} value of 753.7 μgml^{-1} and the highest LC_{50} value of 1148.4 μgml^{-1} was exhibited by glutinous rice stored at 0.85 A_W for 96 days and at 0.98 A_W for 7 days respectively.

Referring to Table 7.8, riceflour extracts shows > 50% mortality after a time period of 7 days at 0.90 and 0.95 A_w and after 26 days when stored at 0.80, 0.85 and 0.98 A_w . The LC_{50} obtained were significant ($p = 0.05$) and the lowest LC_{50} value of 373.4 μgml^{-1} was exhibited by riceflour stored at 0.95 A_W for 54

Table 7.6 Computed probit analyses data (18 hrs) obtained for brine shrimp larvae, tested against ordinary rice extracts stored at different levels of A_w

Water activity	Length of storage (days)	LC_{50} (μgml^{-1})	Chi Sq	Confidence limits	
				Lower	Upper
0.65	7	@			
	26	@			
	54	@			
	96	@			
0.75	7	@			
	26	@			
	54	@			
	96	@			
0.80	7	@			
	26	981.90	2.12*	862.90	1155.90
	54	749.90	0.01*	656.20	854.80
	96	588.10	2.01*	481.60	659.60
0.85	7	@			
	26	433.70	2.50*	385.60	484.70
	54	529.20	1.78*	461.80	612.70
	96	552.30	4.66*	500.70	612.50
0.90	7	@			
	26	741.90	3.35*	656.60	835.60
	54	861.00	3.43*	795.40	935.20
	96	876.20	3.55*	805.50	958.30
0.95	7	@			
	26	325.20	3.86*	288.90	368.40
	54	285.50	0.64*	250.30	326.30
	96	257.20	2.85*	217.00	303.60
0.98	7	@			
	26	854.50	2.35*	780.20	941.30
	54	899.60	1.95*	802.80	1024.80
	96	824.60	1.42*	749.10	911.90

* significant at $p = 0.05$ with 2 degrees of freedom

@ : Inappropriate for probit analysis, < 50% mortality at the highest concentration tested (1400 μgml^{-1}), i.e. considered non-toxic

Table 7.7 Computed probit analyses data (18 hrs) obtained for brine shrimp larvae, tested against glutinous rice extracts stored at different levels of A_w

Water activity	Length of storage (days)	LC_{50} (μgml^{-1})	Chi Sq	Confidence limits	
				Lower	Upper
0.65	7	@			
	26	@			
	54	@			
	96	@			
0.75	7	@			
	26	@			
	54	@			
	96	@			–
0.80	7	@			
	26	972.70	5.22*	896.10	1078.30
	54	876.40	1.91*	801.40	977.50
	96	829.60	2.96*	977.50	883.10
0.85	7	@			
	26	893.30	2.76*	843.50	952.50
	54	983.40	1.67*	836.30	1289.60
	96	753.70	2.03*	679.80	844.50
0.90	7	@			
	26	949.50	0.41*	867.20	1066.10
	54	1009.60	5.52*	925.90	1128.80
	96	1045.20	3.39*	958.70	1170.70
0.95	7	@			
	26	1002.40	5.86*	920.90	1117.20
	54	778.10	2.32*	737.20	823.40
	96	969.60	5.82*	893.60	1074.10
0.98	7	1148.40	0.32*	1038.10	1324.70
	26	784.10	4.42*	704.40	887.80
	54	884.00	1.38*	810.60	982.30
	96	1089.80	4.45*	987.50	1248.40

* Significant at p = 0.05 with 2 degrees of freedom

@ : Inappropriate for probit analysis, < 50% mortality at the highest concentration tested (1200 μgml^{-1}), i.e considered non-toxic

Table 7.8 Computed probit analyses data (18 hrs) obtained for brine shrimp larvae, tested against riceflour extracts stored at different levels of A_W

Water activity	Length of storage (days)	LC_{50} (μgml^{-1})	Chi Sq	Confidence limits	
				Lower	Upper
0.65	7	@			
	26	@			
	54	@			
	96	@			
0.75	7	@			
	26	@			
	54	@			
	96	@			
0.80	7	@			
	26	801.70	3.73*	700.20	923.90
	54	680.30	5.63*	565.50	829.60
	96	718.10	3.57*	600.50	871.20
0.85	7	@			
	26	768.30	2.16*	623.80	975.60
	54	802.70	4.58*	648.20	1031.40
	96	628.10	4.39*	510.50	783.80
0.90	7	1353.00	3.30*	1021.40	2039.70
	26	519.00	2.11*	443.00	604.90
	54	417.90	4.45*	340.90	505.10
	96	406.80	1.92*	324.70	500.80
0.95	7	902.60	1.66*	733.10	1161.30
	26	393.60	0.44*	316.00	481.30
	54	373.40	0.08#	325.60	421.70
	96	468.10	3.56*	383.70	566.40
0.98	7	@			
	26	816.50	2.59*	678.50	1008.00
	54	471.70	2.84*	395.90	557.10
	96	510.10	3.91*	425.20	609.00

* Significant at p = 0.05 with 2 degrees of freedom

\# Significant at p = 0.05 with 1 degree of freedom

@ : Inappropriate for probit analysis, < 50% mortality at the highest concentration tested (1600 μgml^{-1}), i.e. considered non-toxic

days and the highest LC_{50} value of 1353.0 μgml^{-1} was exhibited by riceflour stored at 0.90 A_W for 7 days.

Referring to Table 7.9, glutinous riceflour extracts showed > 50% mortality after a time period of 54 days when stored at 0.80 and 0.85 A_W while glutinous riceflour stored at 0.90 - 0.98 A_W gave >50% mortality earlier i.e. after 26 days. The LC_{50} obtained were significant ($p = 0.05$) and the lowest LC_{50} value of 97.5 μgml^{-1} was exhibited by glutinous riceflour is stored at 0.98 A_W for 54 days and the highest LC_{50} value of 695.9 μgml^{-1} was exhibited by riceflour stored at 0.95 A_W for 96 days.

Referring to Table 7.10, wheatflour extracts showed > 50% mortality when stored at > 0.75 A_w. LC_{50} was obtained after 54 days of storage at 0.75 A_w, after 26 days at 0.80 - 0.95 A_w and earlier at 0.98 A_w i.e. 7 days. The LC_{50} obtained were significant ($p = 0.05$) and the lowest LC_{50} value of 236.7 μgml^{-1} was exhibited by wheatflour stored at 0.90 A_W for 96 days and the highest LC_{50} value of 2297.1 μgml^{-1} was exhibited by wheatflour stored at 0.98 A_W for 7 days.

In Table 7.11, cornflour extracts showed > 50% mortality at 0.85 and 0.90 A_w at 54 and 26 days of storage respectively. The LC_{50} obtained were significant ($p = 0.05$) and the lowest LC_{50} value of 825.4 μgml^{-1} was exhibited by cornflour stored at 0.90 A_W for 54 days and the highest LC_{50} value of 1406.3 μgml^{-1} was exhibited by cornflour stored at 0.85 A_W for 96 days. Cornflour extracts after storage at 0.65 and 0.75 A_W shows < 50% mortality even after 96 days.

Table 7.9 Computed probit analyses data (18 hrs) obtained for brine shrimp larvae, tested against glutinous riceflour extracts stored at different levels of A_W

Water activity	Length of storage (days)	LC_{50} (μgml^{-1})	Chi Sq	Confidence limits	
				Lower	Upper
0.65	7	@			
	26	@			
	54	@			
	96	@			
0.75	7	@			
	26	@			
	54	@			
	96	@			
0.80	7	@			
	26	@			
	54	282.00	4.50*	246.80	319.20
	96	287.40	3.40*	251.80	325.20
0.85	7	@			
	26	@			
	54	265.50	5.62*	232.60	299.60
	96	283.60	0.50*	245.60	324.10
0.90	7	@			
	26	297.00	4.26*	257.10	340.60
	54	416.90	3.16*	338.10	536.60
	96	447.30	4.00*	375.90	550.70
0.95	7	@			
	26	417.40	3.78*	358.30	495.20
	54	320.70	4.82*	277.50	369.80
	96	695.90	3.52*	589.60	879.30
0.98	7	@			
	26	324.30	4.45*	257.40	409.50
	54	97.50	0.42*	49.90	140.20
	96	401.50	5.38*	345.90	472.60

* Significant at p= 0.05 with 2 degrees of freedom.

@ : Inappropriate for probit analysis, < 50% mortality at the highest concentration tested (1200 μgml^{-1}), i.e. considered non-toxic.

Table 7.10 Computed probit analyses data (18 hrs) obtained for brine shrimp larvae, tested against wheatflour extracts stored at different levels of A_w

Water activity	Length of storage (days)	LC_{50} (μgml^{-1})	Chi Sq	Confidence limits	
				Lower	Upper
0.65	7	@			
	26	@			
	54	@			
	96	@			
0.75	7	@			
	26	@			
	54	1890.10	3.97*	1600.30	2393.00
	96	1479.50	0.50*	1332.60	1664.50
0.80	7	@			
	26	1046.90	0.14*	939.00	1164.50
	54	1344.90	4.41*	1183.30	1556.50
	96	1158.80	4.06*	1058.80	1268.50
0.85	7	@			
	26	1086.30	4.08*	986.60	1195.10
	54	1097.70	3.94*	966.70	1249.10
	96	1002.80	2.90*	867.60	1155.10
0.90	7	@			
	26	338.60	2.15*	294.30	392.90
	54	277.60	5.61*	241.70	318.40
	96	236.70	2.80*	203.60	272.70
0.95	7	@			
	26	881.30	0.01*	627.60	1164.60
	54	388.60	4.41*	98.40	612.00
	96	1001.50	0.19*	882.70	1132.10
0.98	7	2297.10	2.79*	1816.70	3399.80
	26	1489.40	5.65*	1339.60	1679.60
	54	1297.50	0.42*	1117.50	1542.20
	96	1131.70	5.24*	969.20	1334.60

* Significant at p = 0.05 with 2 degrees of freedom.

@ : Inappropriate for probit analysis, < 50% mortality at the highest concentration tested (2000 μgml^{-1}), i.e. considered non-toxic.

Table 7.11 Computed probit analyses data (18 hrs) obtained for brine shrimp larvae, tested against cornflour extracts stored at different levels of A_w

Water activity	Length of storage (days)	LC50 (μgml^{-1})	Chi Sq	Confidence limits	
				Lower	Upper
0.65	7	@			
	26	@			
	54	@			
	96	@			
0.75	7	@			
	26	@			
	54	@			
	96	@			
0.80	7	@			
	26	@			–
	54	@			
	96	@			
0.85	7	@			
	26	@			
	54	1307.30	4.47*	1154.80	1525.70
	96	1406.30	0.38*	1230.60	1676.50
0.90	7	@			
	26	1133.60	0.76*	949.70	1414.90
	54	825.40	0.58*	712.70	943.60
	96	1014.80	0.29*	888.00	1170.20
0.95	7	@			
	26	@			
	54	@			
	96	@			
0.98	7	@			
	26	@			
	54	@			
	96	@			

* Significant at p = 0.05 with 2 degrees of freedom.

@ : Inappropriate for probit analyses, < 50% mortality at the highest concentration tested (1800 μg/ml), i.e. considered non-toxic.

7.4 DISCUSSION

Studies by several workers (Wall and Lillehoj, 1983; Lansden, 1984; Hill *et al.*, 1984; Frisvad, 1987) on the HPLC of mycotoxins using a reversed-phase column material was superior to a normal-phase material with regard to separation, peak shape and reversible binding of secondary metabolites to the column. Hence, in this study reversed-phase HPLC was used to detect and separate seven types of mycotoxins in starch-based foods using C_{18} Econosil column with the elution solvent consisting of water and 0.05% trifluoroacetic acid in acetonitrile.

After optimization on the time programme of the gradient and flow-rates of solvents, the system cited in Materials and Methods gave the best results. The seven mycotoxins were eluted in 34 mins and the retention times were highly reproducible with coefficient of variations ranging from 0.3 - 0.7% (Table 7.1). Resolution of the most important aflatoxins G_1 (peak 2) and aflatoxin B_1 (peak 3) was good. Resolution were also good for patulin (peak 1), griseofulvin (peak 4), cytochalasin E (peak 5), ochratoxin A (peak 6) and sterigmatocystin (peak 7). Sterigmatocystin however, gave a broad peak as opposed to the other mycotoxins which gave sharp, narrow and symmetrical peaks (Fig. 7.1). Reproducibility of peak height measurements was good with coefficient of variations ranging from 1.5 - 11.0%.

The detection limits were in nanogram amounts of patulin, griseofulvin aflatoxin B_1, aflatoxin G_1, sterigmatocystin and ochratoxin A. The detection limit of cytochalasin E was poor since only microgram amounts of the toxin can be detected.

The reversed-phase column used did not show any signs of degeneration after 200 runs with chloroform extracts. The HPLC method described here may also be used for multi-mycotoxin screening method in foods.

An evaluation of the extraction method was conducted on artificially contaminated samples at three levels of contamination. Recoveries and the range of coefficients of variation were > 90% and 0.6 - 9.4% for patulin, > 91% and 0.9 - 6.9% for aflatoxin G_1, > 92% and 0.3 - 7.4% for aflatoxin B_1, > 83% and 1.1 - 18% for griseofulvin, > 89% and 0.6 - 3.5% for cytochalasin E, > 88% and 0.8 - 15% for ochratoxin A and > 90% and 1.0 - 11.1% for sterigmatocystin, respectively.

The corresponding quantitation limits, defined as the lowest concentration at which confirmation and reproducible quantitation are possible, were 15 μgkg^{-1} for patulin and griseofulvin, 50 μgkg^{-1} for aflatoxin G_1, B_1 and sterigmatocystin, 75 μgkg^{-1} for ochratoxin A and 2800 μgkg^{-1} for cytochalasin E. These results compare well with those of other methods that show equivalent recoveries (Scott and Hand, 1967; Nesheim *et al.*, 1973; Takeda *et al.*, 1979; Soares and Rodriguez-Amaya, 1985; 1989).

Recoveries were good for all mycotoxins in all six starch-based foods investigated. Hence, being efficient and low cost, this method can be adopted, both for screening and quantitation of large number of samples, with additional savings in time.

The presence of toxigenic species of *Aspergilli* and *Penicillia* on starch-based foods when stored at different levels of A_w predispose the material to mycotoxin formation. The high frequency of *A. flavus* on all the starch-based

foods is responsible for the formation of aflatoxin B_1, aflatoxin G_1 and to some extent sterigmatocystin. *A. flavus* has been known to produce aflatoxins (Cole and Cox, 1981 and Frisvad, 1987;1989) and sterigmatocystin (Frisvad, 1989).

Aflatoxin in general was produced at high levels of relative humidity (87 - 98%) and at moderate temperature (Diener and Davis, 1967). There is no essential relationship between moisture content and aflatoxin formation. Jemmali *et al.* (1969) reported that the moisture content of flour required for growth and aflatoxin production lay between 14 - 16%. In accordance with this study, no aflatoxin was formed in starch-based food below 14% moisture content which corresponds to water activities of approximately 0.75 - 0.80.

The highest concentration of each type of mycotoxin detected occurred at different water activity level for all starch-based food. For example, highest levels of aflatoxin B_1 was detected at 0.80 A_w and 0.95 in ordinary rice and wheatflour but at 0.90 A_w for glutinous rice, riceflour and cornflour and 0.95 A_w for glutinous riceflour. There have been several reports of the interactions between mycotoxigenic species of fungi often leading to a reduction in mycotoxin biosynthesis, but occasionally leading to enhanced production (Boller and Schroeder, 1973;1974). Hesseltine (1967) reported that *A. niger* and *R. arrhizus* growing in competition with *A. flavus* inhibited aflatoxin production.

It should be noted also that the highest concentration of mycotoxins screened were not detected at the highest water activity level (0.98). Although there is no satisfactory explanation to account for this, one possibility is that the faster growing mycelia at higher water activity were capable of breaking down the preformed toxins. There is evidence to support that fungal mycelia are capable of

degrading preformed aflatoxin (Doyle and Marth, 1978). Hence, at higher water activity, fungal mycelia were capable of degrading the preformed toxins at a faster rate than they are being formed due to the highly favourable conditions for growth, and less favourable conditions for toxin production.

Sterigmatocystin formed in rice and wheatflour may be due to the presence of *A. flavus* and *Eurotium repens* on both substrates. Production of sterigmatocystin by *E. repens* has been reported by Frisvad (1989). The formation of patulin in riceflour at 0.95 and 0.98 A_W may be due to the incidence of *A. terreus* or *A. clavatus* and the formation of griseofulvin at $A_W > 0.95$ may be due to *A. versicolor.* The production of patulin by *A. terreus* and *A. clavatus* has been reported previously (Cole and Cox, 1981; Frisvad, 1989; Frisvad and Samson, 1991). *A. versicolor* has also been reported to produce griseofulvin (Frisvad, 1989).

Hence, in this study at 25 °C, there is a tendency for the formation of mycotoxins in starch-based food when exposed to water activities > 0.80. The levels of mycotoxins detected in most instances exceeded the tolerance level established for food in Malaysian legislation (35 μgkg^{-1}). Storage of starch-based foods at high A_w levels not only supported growth of toxigenic fungi, but made possible production of appreciable amounts of mycotoxins. More importantly, toxin production occurred earlier than visible growth i.e. after 26 days of storage at favourable water activities for growth. It would not be surprising to discover toxins especially, aflatoxins in these commodities especially after long term storage, under conducive conditions for fungal growth. Therefore, the control of

environmental conditions is important during storage to inhibit the growth of toxigenic fungi and hence mycotoxin production.

Brine shrimp larvae were found to be a convenient test organism for screening of toxic fungal metabolites. Large numbers of samples can be conveniently screened. Larvae were readily obtained in large numbers, their low natural mortality in dimethyl sulphoxide (DMSO) and the high solubility of food extracts in DMSO made it possible to test crude extracts.

In experiments where > 50% corrected mortality was achieved, a straight-line relationship between probit mortality and log of the concentration of mycotoxin could be demonstrated. LC_{50} values were determined by using the maximum likelihood solution (Finney, 1971) which corrects for natural mortality. Toxicity were not tested at higher concentrations for extracts in which LC_{50} could not be obtained because the percentage mortality of the control (Day 0) extracts showed an increase in percentage mortality as the concentration was increased due to the turbidity of the test samples and also, the amount of extract was not ample for tests at higher concentrations.

The toxicity exhibited by the extracts towards brine shrimp was due to the presence of mycotoxins produced by fungi growing on the substrates. Since the bioassay were conducted on crude extracts, the presence of other toxins not screened for using HPLC may also contribute for the toxicity of the extracts. Scudamore and Hetmanski (1995) observed that in some toxic cereal extracts no mycotoxins screened for were identified. The lack of toxicity by some extracts even though the frequency of occurrence of fungi is high may be due to the

presence of non-toxic strains or the toxins being degraded by competing fungal species.

In this series of experiment, rice, glutinous rice, riceflour and glutinous riceflour exhibited >50% mortality when exposed to water activities of > 0.80 while wheatflour at water activities of > 0.75. Cornflour extracts exhibited > 50% mortality only at 0.85 and 0.90 A_w . Among the starch-based food studied, the highest toxicity was exhibited by glutinous riceflour stored at 0.98 A_w for 54 days with LC_{50} of 97.5 μgml^{-1}, followed by wheatflour stored at 0.90 A_w for 96 days with LC_{50} of 236.7 μgml^{-1}, followed by ordinary rice stored at 0.95 A_w for 96 days with LC_{50} of 257.2 μgml^{-1}, followed by riceflour stored at 0.95 A_w for 54 days with LC_{50} of 373.4 μgml^{-1}, followed by glutinous rice stored at 0.85A_w for 96 days with LC_{50} of 753.7 μgml^{-1} and lastly followed by cornflour stored at 0.90 A_w for 54 days with LC_{50} of 943.6 μgml^{-1}. For long term storage, starch-based foods should be maintained at water activity lower than 0.65 and when exposed to a higher water activity, the storage period should not be more than one month whereby mycotoxins will then be produced.