Effect of Spiking Contact Times on the Analytical Recovery of Aflatoxins

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Summary

Aflatoxins are toxic secondary metabolites of fungi, mainly ***Aspergillus flavus*** *and* ***Aspergillus parasiticus*** *the most common of which are aflatoxins B_1, B_2, G_1 and G_2, and M_1 in milk. Aflatoxin B_1 is normally predominant. The determination of aflatoxins, by extraction, immunoaffinity clean up and liquid chromatography with fluorescence detection is common practice. Recovery correction of the results obtained is mandatory in official analysis for which the only practical approach is separate determination of the analyte added either to aliquots of the sample or matrix blanks, a process commonly referred to as "spiking". Variations in the spiking contact times before extraction could have an effect on the recovery of aflatoxins from the matrix. Herein we describe two studies, a short term (0.5 – 65 hours) and a long term (1 hr - 8 weeks) investigation of the effect of contact time on spike recovery in peanuts, figs and chilli powder. Generally it was found that recovery is dependent upon contact time and this effect is statistically significant for short contact times (less than 24 hours) while thereafter the recovery stabilises. The results from both studies indicated a small effect on contact times in some matrix/aflatoxin/storage condition combinations, however any effect is statistically insignificant compared to the method uncertainty.*

Introduction

Aflatoxins are difuranocoumarin mycotoxins, secondary metabolites produced by fungi, mainly *Aspergillus flavus* and *Aspergillus parasiticus* in or on foods and animal feeding stuffs. Aflatoxins are genotoxic carcinogens[1,2] capable of inducing liver cancer particularly with simultaneous hepatitis B virus infection and are among the most potent mutagens known.[3] Stringent control measures are in place to reduce human consumption, Commission Regulation (EC) No 1881/2006 as amended.[4,5] Aflatoxin B_1 is normally predominant in amount in cultures as well as in food products.

Commission Regulation (EC) No 401/2006[6] lays down methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. The Regulation sets the performance criteria for the analysis of aflatoxins including recommended acceptable ranges of recovery depending on the level of contamination, Table 1, and requires compliance against the limits to be appraised against official analytical control measurements corrected

for analytical recovery. The legislative limits for most food products (apart from baby foods) are between 1-10 µg kg^{-1} where the acceptable recovery range is 70-110%. Recovery must be stated in official reports.

Table 1 – Acceptable Recovery Ranges for Aflatoxins B_1, B_2, G_1 and G_2

Concentration (µg kg^{-1})	Recommended Value
<1.0	50-120 %
1-10	70-110 %
>10	80-110 %

Hence reliable measurement of recovery is essential. Moreover von Holst *et al* [7] showed that in most cases the relative standard deviation calculated from the analysis of Aflatoxin B_1 in various matrices decreased after correction of the results for recovery.

Recovery can be estimated in a number of ways, such as parallel analysis of certified reference materials, inclusion of an isotopically modified version of the analyte or a chemically related internal standard. For practical purposes however aflatoxin recovery estimation is carried out by a separate determination of the analyte added either to aliquots of the sample or matrix blanks, a process commonly referred to as "spiking". Recovery can be influenced by a variety of factors such as the manner in which the analytical method is executed, e.g. extraction procedures, antibody capacity of the immunoaffinity column and the chemical and physical characteristics of the matrix. One of the least tractable problems associated with estimating recovery is that added analyte may not come to effective equilibrium with the native (incurred) analyte.[8] To attempt to address such disequilibrium in casework submitted for technical appeal[9] the authors usually allow a contact time of 14-18 hours (overnight spiking). However to our knowledge the effect of the contact time, (time between spiking and extraction of the analyte), on recovery as a function of the sample matrix has not been investigated. Hence the aim of the project was to assess whether contact time has a significant effect on aflatoxins recovery.

Experiments were built around a statistical plan and aimed to provide information on recoveries obtained at up to seven different contact times. Two studies were carried out. During the first, short-term study the three contact times were: 63-65h, (e.g. spiking on Friday and extracting on the following Monday), 17-19h (e.g. spiking overnight) and 0.5-1h., (e.g. spiking the same day directly before analysis). During the second, long-term study the contact times were: 1 hour, 17 hours, 63 hours, 2 weeks, 4 weeks, 6 weeks and 8 weeks. For peanuts and chillies two long-term storage conditions were tested, frozen and ambient, whilst the figs were stored frozen because of their higher water content and consequent greater vulnerability to the outgrowth of mould and other micro-organisms.

Experimental

The matrices analysed in this project were spiked at their respective legislative limits that were valid at the time of the first study (2011), Table 2. For dried figs the legislative limits were subsequently increased in 2012 by Regulation EU 1058/2012 amending Regulation EC 1881/2006[10].The limits in force prior to 2012 are shown in brackets in Table 2.

Table 2 - Legislative Limits for Aflatoxins

Matrix	Aflatoxin B1 (µg kg^{-1})	Total Aflatoxin (µg kg^{-1})[1]
Chilli Powder	5	10
Peanuts (for human consumption)	2	4
Dried Figs (previous limit in brackets)	6 (2)	10 (4)

1 Total aflatoxin is the sum of aflatoxins B1, B2, G1 and G2

The matrices chosen for the study were skin-on de-shelled peanuts, dried figs and chilli powder (all purchased locally) and are typical of consignments sampled at border inspection posts in the UK and cover three different food categories (nuts, fruits and spices) regulated for their aflatoxin content. Peanuts and figs were homogenised with a defined amount of water to form a slurry prior to analysis while chilli powder was used as received. For peanuts the slurry ratio was 1 part peanuts to 1.4 parts water and for dried figs the ratio was 1 part figs to 1.2 parts water. All the samples were stored frozen and were thawed before spiking.

Initial analysis (six replicates) demonstrated that the peanuts and dried figs did not contain detectable concentrations of aflatoxins (<0.01 µg kg^{-1}). The chilli powder contained aflatoxin B_1 (2.56 ± 0.21 (SD) µg kg^{-1}) and aflatoxin B_2 (0.10 ± (SD) 0.007 µg kg^{-1}) however the repeatability being satisfactory it was decided that the experiment could continue using this material.

Batches and Spiking Concentrations

Analytical batches were prepared in polypropylene bottles. Spiking levels are shown in Table 3.

Table 3 - Spiking Concentrations

	B_1 (µg kg^{-1})	B_2 (µg kg^{-1})	G_1 (µg kg^{-1})	G_2 (µg kg^{-1})
Peanuts	2	1	0.5	0.5
Dried Figs	2	1	0.5	0.5
Chilli Powder	5	2	1.5	1.5

Multiple replicates (7 for the short-term study and 3 for the long-term study) of each matrix were analysed for each time point, together with blank (unfortified) samples and quality control materials. This was repeated three times (total of 21 measurements per contact time per matrix for the short-term study). The analysis was carried out following the same method each time by the same analyst and all the extracts run on the same instrument in order to minimise the factors that contribute to the uncertainty. For statistical purposes a mean contact time was used for each case: 64h, 18h and 1h. Analysis was carried out by ISO 17025 accredited methods[11] established in LGC for many years and consisted of extraction with 60% acetonitrile, (80% methanol for dried figs and chilli powder) followed by immunoaffinity clean up and liquid chromatography with post column derivatisation (Kobra Cell[12]) and fluorescence detection.[13]

Results

Results are shown in the Appendix 1 at the end of the paper. The results for the short-term study are given in tables 4-6, and for the long-term study in tables 7-11.

Discussion

The data were analysed using R[14], which is a widely-used open-source statistical programming system. The data were first inspected for anomalies using scatter plots and box plots and although some potential outliers were observed all data were retained. Data sets contained the following variables: Sample, Batch (of Samples), Matrix, Aflatoxin and Contact Time.

Short-Term Study

Preliminary inspection of the data suggested that for all three matrices there was no evidence of a consistent ordering by sample and little between-batch variability. There was no strong interaction between contact time and aflatoxin, that is, any differences in recovery between the different contact times showed a similar pattern for all four aflatoxins although batch 1 chilli results for aflatoxin G_2 showed more variability than any other batch/aflatoxin across the contact times.

Contact time can be treated in two ways, either as a numerical quantity, using the variable "Time" or as a category. The first would be useful if there were a linear relationship between time and recovery, but in the short-term study there were insufficient contact time values to

tell whether this is the case or not. Contact time was therefore treated as a categorical variable with two degrees of freedom.

Sample and batch are random effects, that is they are treated as random samples from their respective populations, with no specific batch or sample being of interest. The data set was therefore analysed using a linear mixed effects model with maximum likelihood estimation. The model was simplified using a step routine to produce the simplest model which adequately explains the variation in the data (the "minimum adequate model").

The maximal model included sample and batch as random effects and two fixed effects (contact time and aflatoxin) plus an interaction term. For peanuts and chilli powder this was progressively simplified in the same way to produce the minimum adequate model. A model containing only the two fixed effects gave very similar estimates for the fixed effects, showing that between-batch variability is not a significant factor in this data set.

For figs, following the same simplification steps produced a model which retained all terms except a three-way interaction term.

It was found that for peanuts and figs recovery is generally inversely dependent upon contact time and this effect was statistically significant. For chilli powder recovery was weakly dependent on contact time with only borderline statistical significance at one contact time. Recovery of aflatoxins G_1 and G_2 were generally lower than those for aflatoxins B_1 and B_2, a feature of the fact that the immunoaffinity capture has been commercially optimised for aflatoxin B_1.

However the data were complex with variation between batches and samples which was very small compared to the measurement repeatability. In addition, for example in the fig data, recovery also depended on aflatoxin and batch as well as their two-way interactions although this was mainly driven by batch-to-batch variability at contact time 63 to 65 hours. In chilli powder the dependence of recovery on aflatoxin was very strongly significant, with aflatoxins B_1 and B_2 again producing higher recovery than aflatoxins G1 and G2.

Long Term Study

Preliminary assessment of the data suggested differences in recovery between ambient and frozen samples as might be expected since covert outgrowth of mould and aflatoxin production cannot be ruled out in ambient storage. The data also showed large within-day variability compared with change across time as shown in figures 1, 2 and 3.

The data were analysed using classical linear models, with days (time in contact with the sample), condition (ambient, frozen) and matrix as variables. Initially, each aflatoxin was analysed independently. However, the residuals deviated considerably from the assumed normal distribution and it was decided to divide the data into subsets containing one matrix and one aflatoxin. Diagnostic plots (not shown) confirmed that this generated normally

distributed residuals although some subsets showed some anomalous observations and in general Contact Time did not change the recovery significantly.

Figure 1 - Recoveries over Time for Aflatoxin B_1 in Peanuts

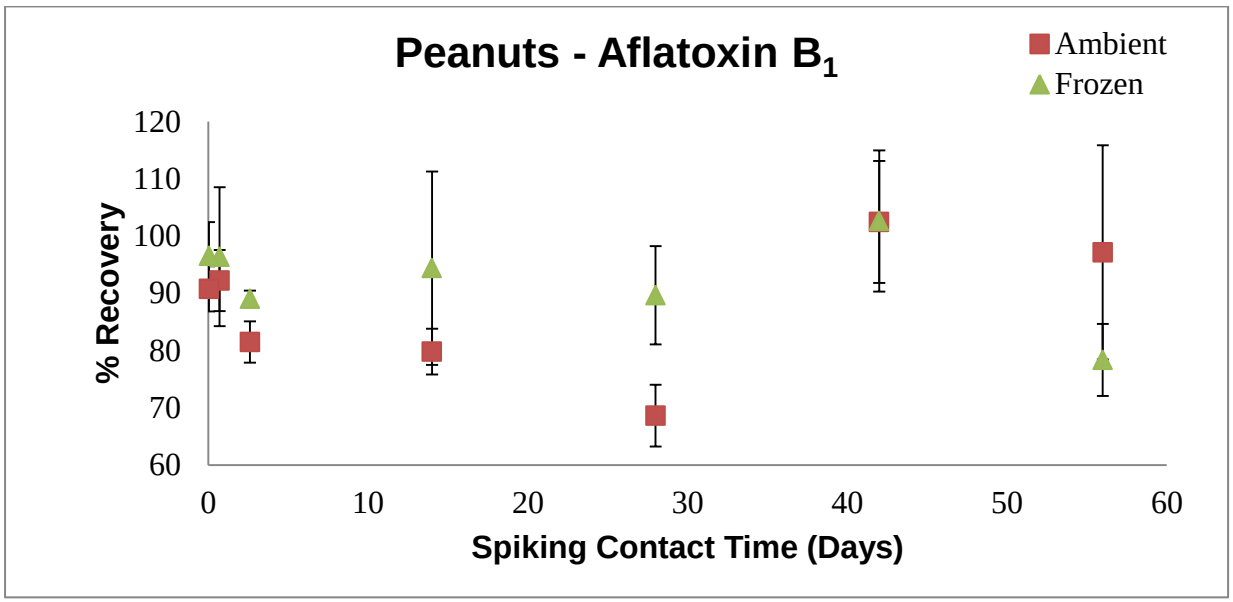


Figure 2 - Recoveries over time for Aflatoxin B_1 in Chilli Powder

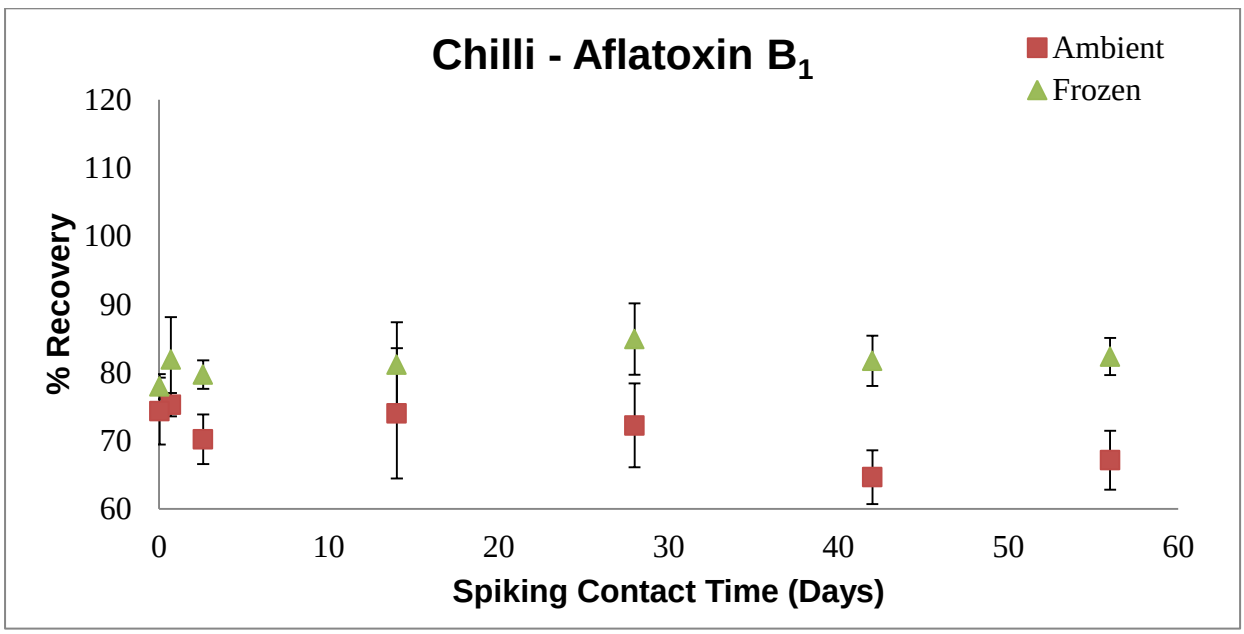
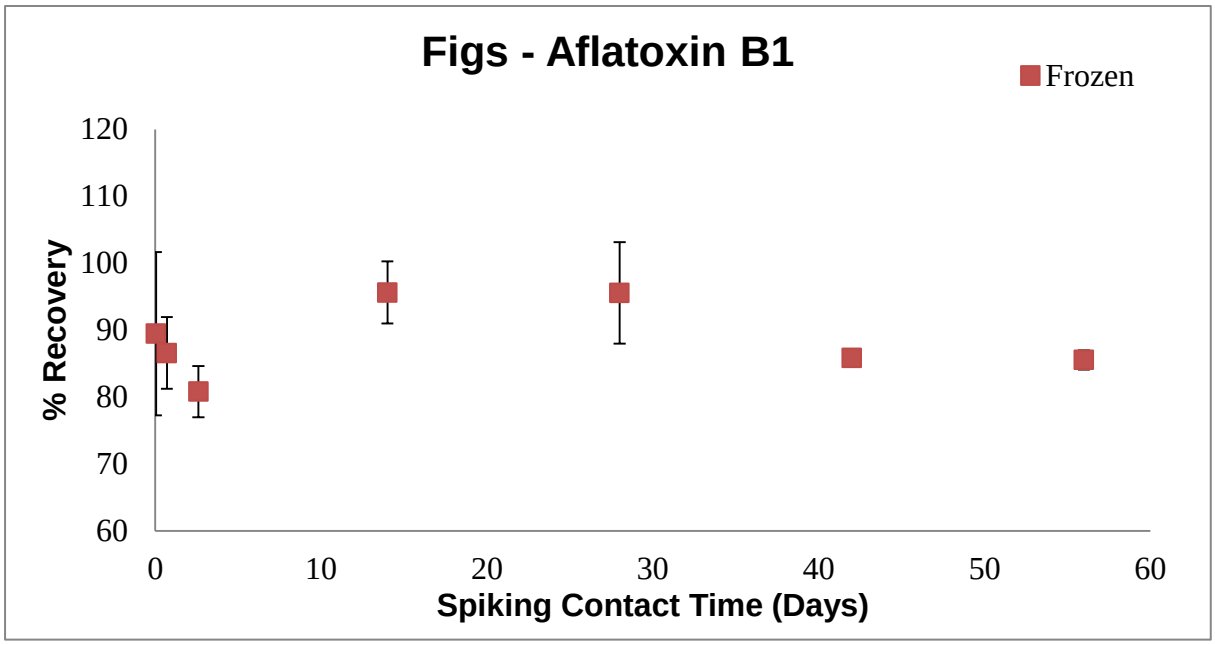


Figure 3 - Recoveries over time for Aflatoxin B_1 in Figs



Conclusions

Overall it can be said that recovery tended to vary inversely with contact time and this effect was statistically significant for short contact times (less than 24 hours) while after 72 hours the recovery stabilised. The results from both studies indicated a small effect on contact times in some matrix/aflatoxin/storage condition combinations, however any effect was statistically insignificant compared to the method uncertainty.

Since there was a reduction in recovery after overnight spiking compared with spiking just prior to analysis we advocate spiking and overnight contact to attempt to account for recovery of incurred aflatoxins for technical appeal (referee) cases. In general this approach should tend to reduce any negative bias in the recovery-corrected results arising from enhanced binding of the native aflatoxins relative to the spiked compounds.

The European Food Safety Authority has advised that reduction of total dietary exposure to aflatoxins could be achieved by reducing the number of *highly contaminated* [our emphasis] foods reaching the market.[15] The dispersion of the results overall, which we believe is typical for analysis for aflatoxins in these and similar matrices at concentrations close to the legislative limits, and the apparent stabilisation of the recovery seen in the long term study in our view outweigh any negative bias that might result in routine analysis by not applying spiking contact times in excess of about one hour. Hence it is not proportionate to recommend spiking contact times longer than about one hour to laboratories carrying out routine official analysis where there is often significant time pressure to produce a result on a cargo incurring demurrage charges and other costs.

It is possible that spiking at higher concentrations of aflatoxins may have produced different findings.

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References

1 European Commission, 1996, Reports of the Scientific Committee for Food, (Thirty-fifth series), available at: http://ec.europa.eu/food/food/chemicalsafety/contaminants/scf_reports_35.pdf (accessed 21.09.2014)

2 Barlow S, Renwick AG, Kleiner J, et al, 2006, Risk assessment of Substances that are both Genotoxic and Carcinogenic, Food and Chemical Toxicology **44**, 1636-1650

3 Herrman JL, and Walker R, 1999, Risk Analysis of Mycotoxins by the Joint FAO/WHO Expert Committee on Food Additives (JECFA), available at: http://www.fao.org/docrep/X2100T/X2100T04.HTM (accessed 21.09.2014)

4 Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting Maximum Levels for Certain Contaminants in Foodstuffs, (OJ L364, 20.12.2006, p5), consolidated version of 01.07.2014, http://eur-lex.europa.eu/legal-content/EN/TXT/?qid=1411320893403&uri=CELEX:02006R1881-20140701 (accessed 21.09.2014)

5 European Commission, Food Contaminants, Aflatoxins, available at: http://ec.europa.eu/food/food/chemicalsafety/contaminants/aflatoxins_en.htm (accessed 21.09.2014)

6 Commission Regulation (EC) No 401/2006 of 23 February 2006 laying down the Methods for Sampling and Analysis of the Official Control of the Levels of Mycotoxins in Foodstuffs, consolidated version 13.03.2010, http://eur-lex.europa.eu/legal-content/EN/TXT/?qid=1411321426493&uri=CELEX:02006R0401-20100313 (accessed 21.09.2014)

7 von Holst C, Stroka J, Anklam E, 2002, Correction of Analytical Results for Recovery: a Comparison of the Method Performance Characteristics from Recent Collaborative Trials Studies for Aflatoxin Quantification using Conventional and Robust Statistics, Food Addit Contam, **19**, 701-708

8 Thompson M, Ellison SLR, Fajgelj A, Willetts P and Wood R, 1999, Harmonised Guidelines for the Use of Recovery Information in Analytical Measurement. Pure and Applied Chemistry, **71**, 337-348

9 Walker MJ, and Gray K, 2013, Quis custodiet – a Review of the Resolution of Disputed Chemical Results in the UK Official Feed and Food Control System 2010-2011, J Assoc Public Analysts (Online) 2013, **41**, 1-27

10 Commission Regulation (EU) No 1058/2012 of 12 November 2012 amending Regulation (EC) No 1881/2006 as regards Maximum Levels for Aflatoxins in Dried Figs, OJ 13.11.2012 L313/14

11 LGC SOP FFF/A1-0315, Determination of Aflatoxins B1, B2, G1 and G2 in Foods, Feedstuffs and Animal and Fish Tissue

12 Papadopoulou-Bouraoui A, Stroka , and Anklam E, 2002, Comparison of Two Post-Column Derivatization Systems, Ultraviolet Irradiation and Electrochemical Determination, for the Liquid Chromatographic Determination of Aflatoxins in Food, JAOAC Int, **85**, 411-416

13 Turner NW, Subrahmanyam S and Piletsky SA, 2009, Analytical Methods for Determination of Mycotoxins: A Review, Analytica Chimica Acta, **632**, 168-180

14 R Core Team (2013). R: A language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria available at: http://www.R-project.org/ (accessed 12.11.2014)

15 Opinion of the Scientific Panel on Contaminants in the Food Chain on a Request from the Commission Related to the Potential Increase of Consumer Health Risk by a Possible Increase of the Existing Maximum Levels for Aflatoxins in Almonds, Hazelnuts and Pistachios and Derived Products, 2007, The EFSA Journal, **446**, 1 - 127

Corrigendum (added 02/02/2015) – Effect of Spiking Contact Times on the Analytical Recovery of Aflatoxins

It has been pointed out that the phrase “method uncertainty” used in this paper may cause confusion with “measurement uncertainty” which is associated with a result rather than with a method. The authors are happy to clarify that for “method uncertainty” we mean the typical dispersion of results obtained in our hands by the method applied to the matrices analysed.

Appendix 1 - Results

Table 4 - Aflatoxin Recoveries from Peanuts

	Average Recoveries (Mean of 7 replicates) - Peanuts (%)											
Contact Time (h)	B_1			B_2			G_1			G_2		
	Batch 1	**Batch 2**	**Batch 3**	**Batch 1**	**Batch 2**	**Batch 3**	**Batch 1**	**Batch 2**	**Batch 3**	**Batch 1**	**Batch 2**	**Batch 3**
64	75.6	75.1	85.2	79.0	78.3	86.7	59.8	61.8	71.9	64.8	67.0	76.4
18	77.7	72.7	78.8	83.5	78.2	78.2	61.8	61.5	68.4	74.8	69.7	74.5
1	81.0	79.2	81.3	89.5	85.1	88.5	71.9	67.5	68.7	73.8	76.6	77.2

Table 5 - Aflatoxin Recoveries from Figs

	Average Recoveries (Mean of 7 replicates) - Peanuts (%)											
Contact Time (h)	**B_1**			**B_2**			**G_1**			**G_2**		
	Batch 1	**Batch 2**	**Batch 3**	**Batch 1**	**Batch 2**	**Batch 3**	**Batch 1**	**Batch 2**	**Batch 3**	**Batch 1**	**Batch 2**	**Batch 3**
64	83.8	89.4	100.7	84.4	88.3	95.7	51.2	53.1	63.2	58.4	64.0	67.3
18	82.5	83.4	83.4	85.9	88.0	93.0	51.6	53.7	57.9	62.9	64.3	63.5
1	83.5	87.2	93.1	88.0	90.6	95.9	54.2	63.2	61.9	65.3	65.0	70.3

Table 6 - Aflatoxin Recoveries from Chilli Powder

	Average Recoveries (Mean of 7 replicates) – Chilli powder (%)											
Contact Time (h)	B_1			B_2			G_1			G_2		
	Batch 1	Batch 2	Batch 3	Batch 1	Batch 2	Batch 3	Batch 1	Batch 2	Batch 3	Batch 1	Batch 2	Batch 3
64	80.3	80.8	79.2	83.2	83.2	77.3	75.5	66.8	61.4	62.0	71.1	68.2
18	80.1	76.4	79.6	81.7	79.1	77.8	73.9	63.2	64.4	65.7	69.8	69.7
1	79.0	75.3	86.0	81.3	81.3	81.5	73.7	65.0	67.6	71.6	71.3	71.6

Table 7 - Aflatoxin Recoveries from Chilli Powder stored at Ambient

Chilli Powder Ambient		% Recovery (Mean of 3 replicates)			
Weeks / Hours	**Spiking time (Days)**	**B_1**	**B_2**	**G_1**	**G_2**
8 Weeks	56	67.16	77.67	59.61	63.46
6 Weeks	42	64.67	69.38	58.07	59.34
4 Weeks	28	72.26	71.32	58.66	61.63
2 Weeks	14	74.04	79.96	63.57	64.14
63 hours	2.6	70.21	76.47	62.47	54.91
17 hours	0.7	75.29	81.34	67.33	75.95
1 hour	0.04	74.35	75.99	61.27	92.29

Table 8 - Aflatoxin Recoveries from Chilli Powder stored Frozen

Chilli Powder Frozen		% Recovery (Mean of 3 replicates)			
Weeks / Hours	**Spiking time (Days)**	**B_1**	**B_2**	**G_1**	**G_2**
8 Weeks	56	82.35	81.41	74.78	131.75
6 Weeks	42	81.71	81.01	63.71	93.18
4 Weeks	28	84.91	79.81	59.67	83.79
2 Weeks	14	81.15	77.95	70.15	71.61
63 hours	2.6	79.68	79.28	63.61	84.83
17 hours	0.7	81.90	80.43	65.30	75.53
1 hour	0.04	76.22	78.68	68.56	73.26

Table 9 - Aflatoxin Recoveries from Slurried Peanut stored at Ambient

Peanuts Ambient		% Recovery (Mean of 3 replicates)			
Weeks / Hours	**Spiking time (Days)**	**B_1**	**B_2**	**G_1**	**G_2**
8 Weeks	56	118.74	79.24	116.91	91.27
6 Weeks	42	118.69	83.41	117.75	96.09
4 Weeks	28	68.62	65.51	78.43	62.87
2 Weeks	14	79.81	69.67	73.19	63.71
63 hours	2.6	81.48	73.60	75.15	61.65
17 hours	0.7	92.22	80.99	81.28	68.81
1 hour	0.04	95.22	80.02	73.02	65.16

Table 10 - Aflatoxin Recoveries from Slurried Peanut stored Frozen

Peanuts Frozen		% Recovery (Mean of 3 replicates)			
Weeks / Hours	**Spiking time (Days)**	**B_1**	**B_2**	**G_1**	**G_2**
8 Weeks	56	78.33	74.87	125.30	83.15
6 Weeks	42	102.63	59.08	122.89	71.20
4 Weeks	28	96.10	69.26	145.19	68.62
2 Weeks	14	94.37	71.53	120.52	82.97
63 hours	2.6	100.76	83.24	147.20	74.57
17 hours	0.7	135.85	68.75	121.48	82.36
1 hour	0.04	96.51	79.37	153.31	80.03

Table 11 - Aflatoxin Recoveries from Slurried Figs stored Frozen

Figs Frozen		% Recovery (Mean of 3 replicates)			
Weeks / Hours	**Spiking time (Days)**	**B_1**	**B_2**	**G_1**	**G_2**
8 Weeks	56	85.56	84.93	70.33	76.42
6 Weeks	42	85.88	83.73	74.36	79.89
4 Weeks	28	95.57	87.27	84.06	85.47
2 Weeks	14	95.62	91.36	81.86	79.65
63 hours	2.6	80.84	82.76	80.69	76.84
17 hours	0.7	86.60	90.50	93.34	87.19
1 hour	0.04	89.49	91.79	125.43	78.89