Aflatoxins in Groundnuts – Assessment of the Effectiveness of EU Sampling and UK Enforcement Sample Preparation Procedures

Michael Walker[a,b], Peter Colwell[a], Simon Cowen[a], Stephen LR Ellison[a], Kirstin Gray[a], Selvarani Elahi[a], Peter Farnell[a], Phillip Slack[a] and D Thorburn Burns[c]

a Government Chemist Programme, LGC, Queens Road, Teddington, Middlesex TW11 OLY
b To whom correspondence should be addressed, michael.walker@lgcgroup.com
c Institute for Global Food Security, The Queen's University of Belfast, Belfast BT9 5HN

Summary

In the UK the Government Chemist is required to act as the national focus of technical appeal in specified areas where there is an actual or potential dispute between food businesses and regulators on the results of chemical analysis or their interpretation.

Many such disputes have involved aflatoxin results. A European Union regulation controls official methods of sampling, sample preparation (often by high speed slurrying with water) and analysis for mycotoxins in foodstuffs. In view of the known distribution heterogeneity of aflatoxins in food, work was undertaken to investigate the effectiveness of the EU sampling protocol, UK enforcement sample preparation procedures and slurry ratio on the determination of aflatoxins in a lot of groundnuts (peanuts) in shell.

Following six replicate sampling exercises each laboratory set of samples (enforcement, defence and reference) was analysed in a single laboratory for aflatoxins in a manner suitable for detailed statistical interpretation. The results obtained in this study demonstrate that the EU sampling protocol is effective and that when the protocol is properly followed the mean results for the three laboratory samples derived from the sampling exercise are expected to be equivalent.

Introduction

Aflatoxins are toxic metabolites produced by certain strains of the fungi Aspergillus *flavus* and Aspergillus *parasiticus* in or on foods and animal feeding stuffs. They have been associated with various diseases, such as aflatoxicosis, in livestock, domestic animals and humans throughout the world. Aflatoxins have potent carcinogenic effects in susceptible laboratory animals and acute toxicological effects in humans[1]. For example in April 2004, a large aflatoxicosis outbreak occurred in Kenya resulting in 317 cases and 125 deaths[2,3]. Some aflatoxins have been classified by the Scientific Committee on Food (SCF) of the European Commission as genotoxic carcinogens[4]. To minimise the risks from exposure to

aflatoxins many countries have imposed regulatory limits on commodities intended for use as food and feed.

Regulatory control of aflatoxins in foods in the European Union (EU) is by Commission Regulation (EC) No 1881/2006[5], as amended, setting maximum levels for certain contaminants in foodstuffs and implemented in England at the time of writing by the Contaminants in Food (England) Regulations 2013 (equivalent legislation specific to each of the other countries of the UK is also in place). The concentration of aflatoxin B1 in animal feeding-stuffs are controlled by the Animal Feed (Composition, Marketing and Use) (England) Regulations 2015 (again equivalent legislation specific to each country of the UK is also in place) implementing Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed. By way of illustration the Regulations limit the concentrations of aflatoxin B1 and total aflatoxins in groundnuts (peanuts), intended for direct human consumption or as an ingredient in foodstuffs, to maxima of 2.0 micrograms per kilogram and 4.0 micrograms per kilogram respectively. For groundnuts to be subjected to sorting or other physical treatment before human consumption or use as an ingredient in foodstuffs the regulations limit the concentrations of aflatoxin B1 and total aflatoxins to maxima of 8.0 micrograms per kilogram and 15.0 micrograms per kilogram respectively.

All groundnuts consumed in the UK are imported, thus the most efficient means of securing consumer protection from excessive concentrations of aflatoxins from groundnuts is to regulate consignments at import. In practice this results in sampling from static lots by officers of a Port Health Authority followed by sample preparation and analysis at an official food control laboratory (Public Analyst's laboratory)[6]. The analytically measured aflatoxin concentrations are appraised against the regulatory limits, a form of "acceptance" sampling[7].

The potential inhomogeneity of distribution of damaged and mouldy groundnut kernels in a lot, the considerable variation in the aflatoxin concentrations in the affected kernels and the difficulties presented by sampling from static lots are well known[8,9]. These problems are best addressed by appropriate sampling plans, defined by the number of sampling units, the size of each sampling unit, the sample preparation method, the analytical method and the sample acceptance limit[10]. Even so, incorrect decisions are possible. False negatives (a bad lot accepted, associated with "buyers risk") and false positives (a good lot rejected, associated with "sellers risk") carry risks to human health and economic consequences respectively. For a given sampling plan and an assumed aflatoxin distribution it is possible to plot the probability P(M) of acceptance of a lot with an aflatoxin concentration M against M, known as an operating characteristic (OC) curve[11,12]. The OC curve indicates the magnitudes of the buyers and sellers risks. Historically, studies by Whitaker and colleagues have been the most prominent in relating sampling regimes to acceptance/rejection criteria for mycotoxins[13] and they have produced regular updates, since 2009, on developments in mycotoxin analysis[14,15] and a monograph on sampling procedures to detect mycotoxins in a variety of commodities[16]. However, a somewhat different approach has been advocated, for example by Thompson and Fearn[17], who began the development of optimal strategies for apportioning resources between sampling and analysis by a definition of fitness for purpose based on minimising expected downstream possible financial loss. In an innovative and powerful mathematical approach

Ramsey *et al*[18] described an optimised uncertainty method balancing the uncertainty of measurements (separately, sampling and analysis) on food against their cost and the other expenditure that may arise as a consequence of the possible misclassification of the food. Fearn *et al*[19] extended the concept using decision theory but only gave worked examples from non-food areas.

Evaluation of the sampling plans in use in the United States, United Kingdom, and the Netherlands for aflatoxins in shelled groundnuts in the mid-1990s noted that each country prescribed different numbers of sampling units, sampling unit weights and acceptance limits. The consequences were different levels of acceptance or rejection of good and bad lots and a plea for standardisation of mycotoxin sampling procedures[20]. Detailed procedures for sampling, sample preparation and analysis are now laid down in European law and guidance provided for a uniform approach throughout all the member states of the EU[21,22].

However, as far as the authors are aware, there has been no appraisal of the effectiveness of the EU sampling protocols and sample preparation guidelines published in the open literature. Indeed little information is available in the literature regarding the origin or scientific basis of general sampling plans for mycotoxins in food[13].

An avenue of technical redress against potentially incorrect regulatory decisions on acceptance/rejection of food exists in the UK where the Government Chemist is required to act as the national focus of technical and interpretative appeal in specified areas[23]. EU and UK law[6,24,25] requires that a second portion of a formal regulatory sample must be available if the food owner chooses to challenge a regulatory classification. To resolve any dispute occasioned by such a challenge, in the UK, a third portion of the sample is available for a reference analysis by the Government Chemist. Disputed aflatoxin results have been a relatively frequent cause of referral to the Government Chemist. In a number of instances the Government Chemist's findings indicated significant differences between the results obtained by reputable laboratories following their application of the EU-recommended sampling protocol. In the light of these findings and given the known distribution of variability between sampling, sample preparation and analysis it was decided to investigate the effect of sampling within a consignment and differences that can arise from procedures in the sample preparation laboratory.

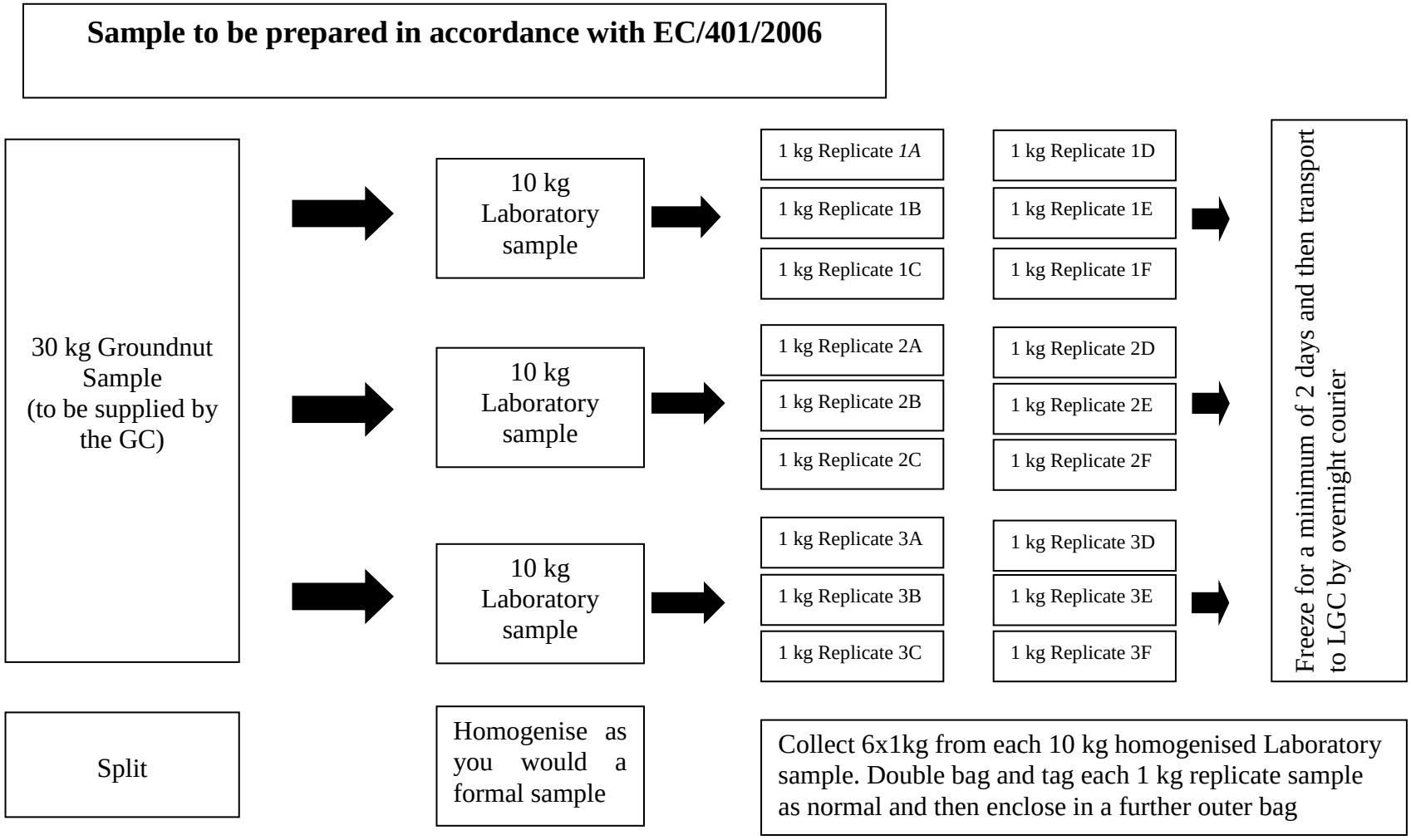
Reduction of the overall variability of a sampling plan can be achieved by increasing the sample and sub-sample sizes and the degree of comminution of granular materials[12]. The EU sampling procedure can result in bulk sample sizes of up to 30kg and in looking at means of taking representative analytical aliquots from these, methods of sample preparation have been investigated[26-29]. It was concluded that sample comminution was best performed by high speed slurry mixing, which produced smaller particles than by dry comminution and, consequently, more homogeneous samples with lower between-sample coefficients of variation. It has also been suggested that slurry mixing diminishes aflatoxin/matrix binding thus enhancing their analytical recovery. Since different laboratories in the UK were known to use different slurry ratios an investigation has been carried out of the effect of slurry ratio on aflatoxin recovery. The overall aims were to assess if the EU legislative procedures are capable of achieving the required level of homogeneity under normal sampling conditions

and to establish whether we can reasonably expect the same interpretation from different laboratories when analysing replicate test portions from the enforcement, defence and reference portions of a formal sample.

The EU law and guidance[21,22] that provide detailed procedures for regulation of aflatoxins contain certain definitions that differ to some extent from those that are used in the technical literature of sampling. The regulation terms are used throughout this paper in order to aid interpretation of the findings in the light of the regulatory protocol. Thus, for ease of reference, "lot" means an identifiable quantity of a food commodity delivered at one time and determined by the official to have common characteristics, such as origin, variety, type of packing, packer, consignor or markings; "sublot" means a designated part of a large lot in order to apply the sampling method on that designated part; each sublot must be physically separate and identifiable; "incremental sample" means a quantity of material taken from a single place in the lot or sublot; "aggregate sample" means the combined total of all the incremental samples taken from the lot or sublot and "laboratory sample" means a sample intended for the laboratory. The term "replicate test portion" is used to denote the homogenised aliquot (usually around one kg) resulting from slurry mixing of the 10kg laboratory sample.

The regulation at the time this study was carried out required increments taken from a lot to be combined into an aggregate sample (up to 30kg) which is then mixed and divided into up to three laboratory samples of 10kg each. Each 10kg laboratory sample must be homogenised (in the UK by high speed slurrying with water), and three replicate test portions must be taken from it. One of these is for analysis by the regulatory (enforcement) laboratory, a second must be available for analysis if the food owner chooses to commission their own analysis and a third is held for a reference analysis by the Government Chemist, if required in cases of a dispute. For clarity if a 30kg aggregate sample is taken each laboratory will receive 3 subsamples, one from each of the 3x10kg sample. Figure 1 describes this procedure[6] as carried out for these experiments. In practice this sub-sampling is carried out by an Official Food Control Laboratory (in the UK a Public Analyst's laboratory). Thus each of the 3 laboratories that carry out the analyses would receive three replicate test portions, one from each of the original 3 homogenised 10kg laboratory samples. The study reported here tests the hypothesis that homogenisation is effective and results for each 10kg laboratory sample should agree across laboratories. On analysis the regulations require that the original lot be rejected if one or more of the laboratory samples (and hence any one of the replicate test portions) exceeds the maximum regulatory limit beyond reasonable doubt taking into account a correction for recovery and measurement uncertainty.

Figure 1 - Generation of 18x1kg Sub-samples by Each Public Analyst Laboratory for Analysis at the Laboratory of the Government Chemist (GC) from each of two 30kg Lots



Experimental

Sampling and Sample Preparation

A suitable lot was identified when 54x30kg bags (1620kg in total) were left behind at a port from a rejected consignment of in-shell groundnuts. The smallest lot for which EU law specifies the production of a 30kg aggregate sample is 15 tonnes. However 1.6tonnes was judged a suitable quantity from which to assess sampling procedures since it originated from a larger consignment that had already been found to be non-compliant. Six separate sampling exercises following the recommended EU sampling protocol were undertaken from this 1.6tonne lot of in-shell groundnuts; three by a Port Health Authority (PHA) officer and three by Government Chemist (GC) staff, resulting in six separate 30kg aggregate samples.

Each sampling exercise was carried out by opening each sack in the consignment and manually removing an incremental aliquot so that the bulked incremental aliquots provided an aggregate sample weighing approximately 30kg. The increments were taken from each sack randomly so that no aggregate sample contained groundnuts from only one part (top, middle or bottom) of the sack.

Three UK Official Food Control (Public Analysts, PA) laboratories were identified with facilities for and experience in preparation of samples for aflatoxin determinations by the EU recommended sample preparation procedures (high speed slurrying with water). The six 30kg aggregate samples prepared from the 1.6tonne lot were distributed among the three participating public analyst laboratories, each receiving one 30kg sample prepared by the PHA Officer and one 30kg sample prepared by GC staff. Each PA laboratory was asked to use its own methods (in accordance with EC/401/2006) to mix each 30kg aggregate sample and divide it into the required three 10kg laboratory samples. Each PA laboratory was also asked to use their own method of sample preparation to prepare the required three 1kg replicate test portions by high speed slurrying with water (for enforcement, food business and referee respectively) from each 10kg laboratory sample.

The three participating PA laboratories employed similar procedures. Two of the three laboratories used cement mixers to tumble each 30kg aggregate sample of groundnuts prior to splitting into 10kg laboratory samples, see Figure 2. One laboratory used a manual “coning and quartering” procedure to divide the 30kg aggregate sample into three 10kg laboratory samples.

Figure 2 Bulk Sample Mixing using a Cement Mixer (courtesy Kent Scientific Services)

The 10kg laboratory samples were homogenised with water in mass ratios that varied from 1 part groundnuts to 2.4 parts water to 1 part groundnuts to 3.0 parts water using high speed rotating blade blenders, specifically, Silverson stainless steel High Shear Batch Mixer with stand, model DX with slotted disintegrating head, see Figure 3, in two laboratories, and a more powerful Silverson model EX mixer powered by 4HP, 3000rpm, TEFV/IP55 motor, 415/3/50. 3 phase in the third laboratory. Government Chemist staff witnessed preparation of the slurries at the three laboratories.

Figure 3 Slurrying with Water
(courtesy Kent Scientific Services)

As a precaution the procedures were carried out in duplicate (although in the event only one set of the duplicates was analysed) and all the prepared replicate test samples were deep frozen and forwarded to the Government Chemist. Figure 1 shows the diagram that was sent to the sample preparation laboratories as part of their instructions.

Analysis

The 54 replicate test samples arising from the 18 laboratory samples were analysed in duplicate at LGC, resulting in a total of 108 observations. These 108 measurements were obtained in nine separate runs (12 measurements per run).

One CRM (BCR-264, defatted peanut meal) in duplicate, one blank sample, and one duplicate spike recovery check sample were added to each run. To the extent possible, a balanced blocked allocation of samples to runs was used to minimise the effect of between-run variation on any test for the sample preparation laboratory, aggregate 30kg sample and laboratory sample effects. Four samples from each sample preparation laboratory – two per sampler (PHA or GC) were included in each analytical run, balancing sample preparation laboratory and sampler effect across runs. Further, two samples from each of two laboratory samples per lab were included in each run, forming a balanced incomplete block arrangement for laboratory sample. The run order was then randomised individually for each run and the recoveries of aflatoxins determined. Control samples and blanks were prepared at the ratio 1 part groundnut to 2.5 parts water for all batches.

Aflatoxins were extracted from 50g of slurry with acetonitrile/water 60/40 v/v (adjusted for the amount of water added in slurrying). The amounts of groundnuts-in-shell represented by 50g of slurry varied from 12.5g to 14.7g as the nut:water ratio varied from 1:3 to 1:2.4. The extract was cleaned by immunoaffinity column using R-Biopharm Easi-extract® aflatoxins cartridges[30]. The determinations of aflatoxins B1, B2, G1 and G2 were made by liquid

chromatography, duplicate injections with post-column derivatisation and fluorescence detection according to an accredited procedure specifying, the column Spherisorb 5µm ODS2 (25cm 4.5mm) or equivalent; mobile phase: 1080mL water, 580mL methanol and 340mL acetonitrile were mixed and 238mg KBr and 700µL 4N HNO_3 were added and thoroughly mixed. Detection was by fluorescence after electrochemical derivatisation (Kobra cell). All analysis was carried out in subdued light.

For the investigation of slurry ratio effect on recovery and to choose a suitable ratio for preparation of control samples (unfortified and fortified blanks) known concentrations of aflatoxins were added to replicate samples of slurried blank material (groundnuts shown by previous analysis to be free from significant quantities of aflatoxins) prepared at each of the slurry ratios used by the participating laboratories.

All statistical analysis was performed using R version 2.6.0[31,32].

Results and Discussion

Effect of Slurry Ratio on Extraction

All aflatoxin data reported herein have been corrected for mean blank responses and appropriate recovery. The observed recoveries for the different aflatoxins at each slurry ratio are listed in Table 1. Two-way analysis of variance was very strongly significant ($p<0.001$) for effects for the aflatoxin, the slurry ratio, and the aflatoxin:slurry ratio interaction. Recoveries therefore differ significantly between aflatoxins and between slurry ratios, and the slurry ratio effect is not consistent across different aflatoxins.

Table 1 – Recovery Data Obtained for Varying Slurry Ratios

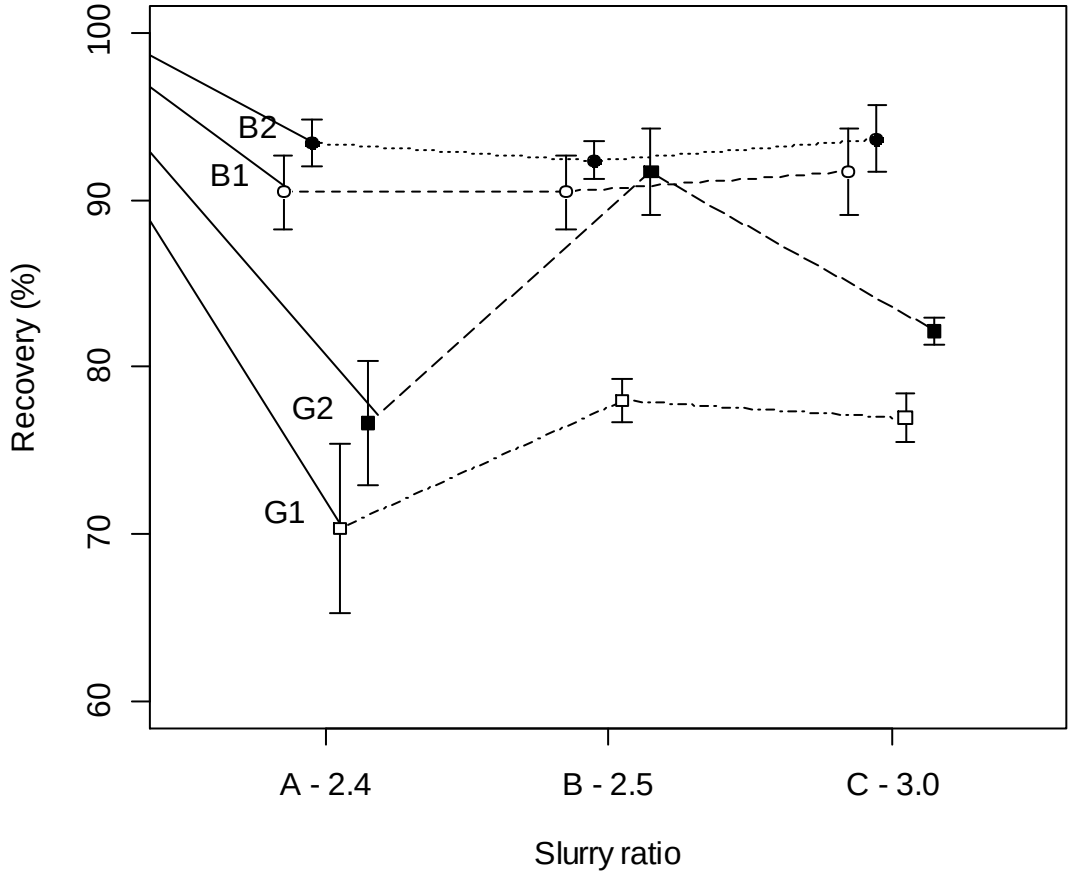
	Sample A - 1 Part Groundnut to 2.4 Parts Water				**Sample B - 1 Part Groundnut to 2.5 Parts Water**				**Sample C - 1 Part Groundnut to 3.0 Parts Water**			
	Recovery %				**Recovery %**				**Recovery %**			
	B1	**B2**	**G1**	**G2**	**B1**	**B2**	**G1**	**G2**	**B1**	**B2**	**G1**	**G2**
Spike 1	88.9	92.1	70.9	75.8	90.6	91.7	75.4	77.6	89.6	92.7	76.3	82.7
Spike 2	89.7	94.1	66.6	75.3	89.0	91.8	71.7	79.1	91.3	92.6	78.3	81.7
Spike 3	91.4	93.5	69.5	75.3	90.0	93.0	71.7	77.2	92.5	94.2	76.5	82.5
Spike 4	91.9	93.8	74.3	80.1	92.3	93.0	76.0	78.1	93.4	95.2	76.7	81.7
Mean Recovery	90.5	93.4	70.3	76.6	90.5	92.4	73.7	78.0	91.7	93.7	76.9	82.1
Std Dev	1.4	0.9	3.2	2.3	1.4	0.7	2.3	0.8	1.7	1.3	0.9	0.5

This is clear from Figure 4, which shows the mean and 95% confidence intervals for each aflatoxin:slurry ratio combination. It is also clear from the figure that while recoveries for

aflatoxins B1 and B2 are essentially constant between 90% and 95%, recoveries for aflatoxins G1 and G2 appear lower, much more variable and with a possible tendency to increase with slurry ratio. The trend in aflatoxin G recoveries was not consistent in this particular experiment and, since the results reflect a single slurrying operation in each case, may reflect variations in the slurrying process as much as a consistent trend. Nonetheless, it is clear that this part of the process contributes appreciable variability for aflatoxins G1 and G2, and merits further study.

For the purpose of the present study, it suffices to note that the B1 and B2 recoveries are essentially constant with slurry ratio and since these compounds (particularly aflatoxin B1) dominate the total aflatoxin concentration it is reasonably appropriate to discuss the effects of sampling primarily in terms of the sampling variation in aflatoxin B1 and total aflatoxin.

Figure 4: Aflatoxin Recovery at Different Slurry Ratios



The figure shows the mean and 95% confidence interval for recovery for four aflatoxins at three different slurry ratios. Line and symbol styles differentiate results for the different aflatoxins as shown by the text label to the left of each line. Bars show the 95% confidence intervals, calculated from the observed dispersion for each group.

Sampling Effects

For clarity, two samplers (PHA and GC) each sampled 3x30kg "aggregate samples" from the lot. Three sample preparation laboratories received a pair of aggregate samples and prepared 3x10kg "laboratory samples" from each. They then prepared three "slurried replicate test samples" from each laboratory sample for analysis. All the analyses were performed by the Laboratory of the Government Chemist, LGC. The results of the analysis after sorting back from the randomised analysis plan into laboratory order and corrected for daily recovery are presented in Table 2.

Table 2 – Data Obtained Sorted by Consignment (30kg Aggregate)

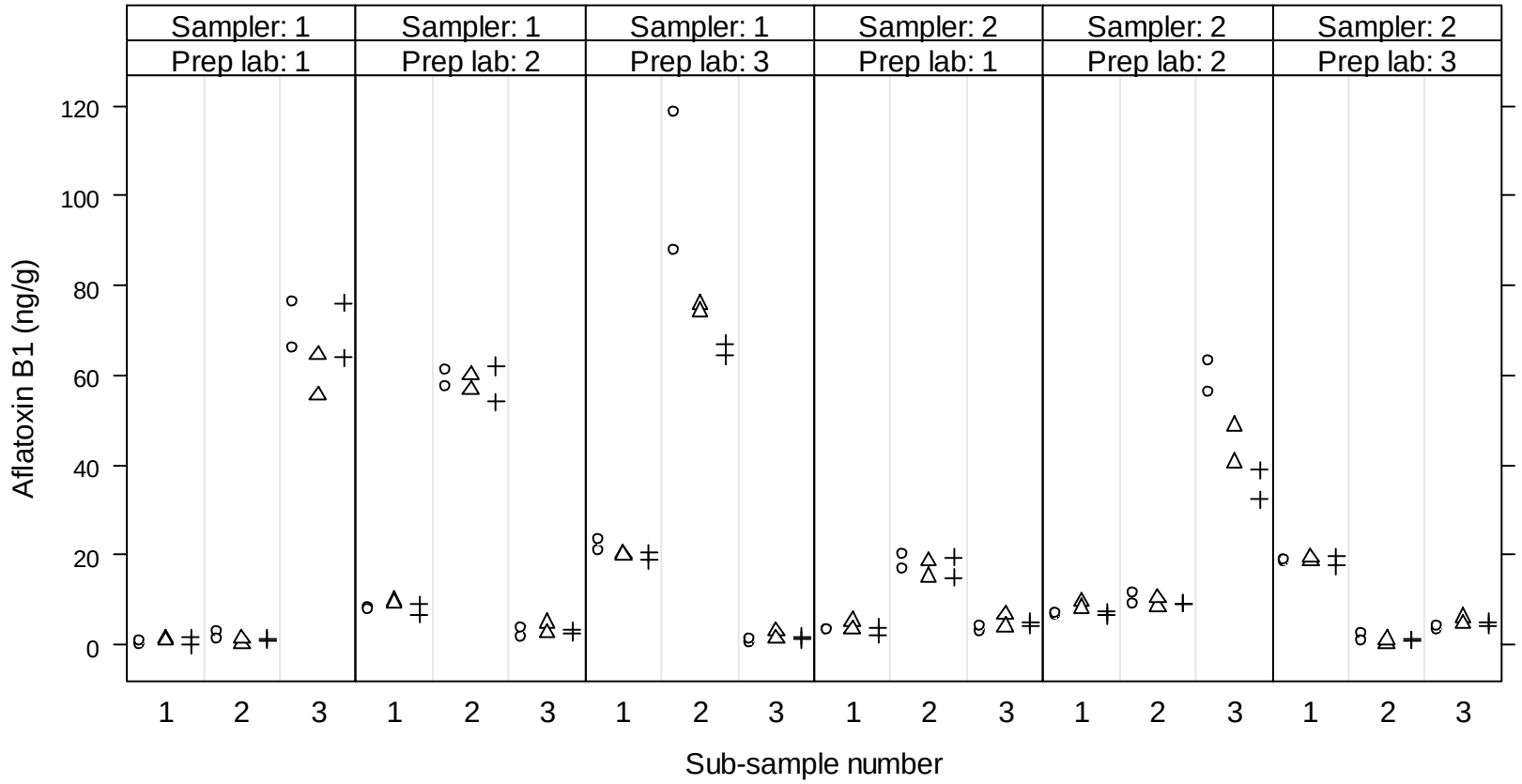
Lab	Consignment Number	10kg Sub-sample Number	Replicate Number	Run/ Batch	LIMS number	B1 (ng/g)	B2 (ng/g)	G1 (ng/g)	G2 (ng/g)	Total
1	1 (PHA)	1	A	1	H3000682	0.18	0.04	0.00	0.00	0.22
1	1 (PHA)	1	A	3	H3000682	1.01	0.00	0.00	0.00	1.01
1	1 (PHA)	1	B	7	H3000683	1.45	0.39	0.00	0.00	1.84
1	1 (PHA)	1	B	9	H3000683	0.89	0.30	0.00	0.00	1.19
1	1 (PHA)	1	C	4	H3000684	1.52	0.35	0.00	0.28	2.15
1	1 (PHA)	1	C	5	H3000684	-0.05	0.19	0.00	0.00	0.14
1	1 (PHA)	2	A	2	H3000688	2.89	0.39	0.00	0.00	3.29
1	1 (PHA)	2	A	4	H3000688	1.24	0.29	0.00	0.00	1.52
1	1 (PHA)	2	B	1	H3000689	0.12	0.01	0.00	0.00	0.12
1	1 (PHA)	2	B	8	H3000689	1.36	0.44	0.00	0.00	1.80
1	1 (PHA)	2	C	6	H3000690	1.03	0.08	0.00	0.00	1.11
1	1 (PHA)	2	C	7	H3000690	1.33	0.37	0.63	0.36	2.69
1	1 (PHA)	3	A	5	H3000694	76.36	30.01	20.36	6.13	132.86
1	1 (PHA)	3	A	6	H3000694	66.29	28.41	13.37	4.98	113.04
1	1 (PHA)	3	B	2	H3000695	64.59	21.61	12.69	5.46	104.35
1	1 (PHA)	3	B	3	H3000695	55.44	22.81	12.38	5.09	95.71
1	1 (PHA)	3	C	8	H3000696	63.93	26.18	11.96	4.83	106.90
1	1 (PHA)	3	C	9	H3000696	75.99	35.67	16.98	6.13	134.77
1	2 (LGC)	1	A	1	H3000700	3.46	1.28	0.00	0.00	4.73
1	2 (LGC)	1	A	3	H3000700	3.42	0.92	0.00	0.00	4.34
1	2 (LGC)	1	B	7	H3000701	5.20	1.59	0.00	0.00	6.79
1	2 (LGC)	1	B	9	H3000701	3.42	1.30	0.00	0.00	4.73
1	2 (LGC)	1	C	4	H3000702	3.73	1.27	0.00	0.00	5.00
1	2 (LGC)	1	C	5	H3000702	2.25	1.04	0.00	0.00	3.28
1	2 (LGC)	2	A	2	H3000706	20.15	2.82	2.98	0.92	26.86
1	2 (LGC)	2	A	4	H3000706	16.97	2.90	2.59	0.85	23.31
1	2 (LGC)	2	B	1	H3000707	15.09	2.51	1.82	0.41	19.83
1	2 (LGC)	2	B	8	H3000707	18.50	3.06	1.68	0.65	23.89
1	2 (LGC)	2	C	6	H3000708	14.98	2.54	1.50	0.42	19.44
1	2 (LGC)	2	C	7	H3000708	19.40	2.96	2.01	0.73	25.10
1	2 (LGC)	3	A	5	H3000712	3.05	0.69	0.00	0.00	3.74

Lab	Consignment Number	10kg Sub-sample Number	Replicate Number	Run/ Batch	LIMS number	B1 (ng/g)	B2 (ng/g)	G1 (ng/g)	G2 (ng/g)	Total
1	2 (LGC)	3	A	6	H3000712	3.94	0.60	0.00	0.00	4.54
1	2 (LGC)	3	B	2	H3000713	6.72	0.97	0.00	0.00	7.69
1	2 (LGC)	3	B	3	H3000713	4.01	0.51	0.00	0.00	4.52
1	2 (LGC)	3	C	8	H3000714	5.11	1.07	0.00	0.00	6.18
1	2 (LGC)	3	C	9	H3000714	4.19	0.87	0.40	0.34	5.80
2	1 (PHA)	1	A	1	H3000752	8.35	0.91	0.00	0.00	9.25
2	1 (PHA)	1	A	3	H3000752	7.74	0.74	0.00	0.00	8.48
2	1 (PHA)	1	B	7	H3000753	9.71	1.08	0.00	0.00	10.79
2	1 (PHA)	1	B	9	H3000753	9.26	1.17	0.00	0.00	10.43
2	1 (PHA)	1	C	4	H3000754	8.85	1.08	0.00	0.00	9.93
2	1 (PHA)	1	C	5	H3000754	6.77	0.84	0.00	0.00	7.61
2	1 (PHA)	2	A	2	H3000758	61.08	11.64	0.00	0.00	72.72
2	1 (PHA)	2	A	4	H3000758	57.55	13.72	0.00	0.00	71.27
2	1 (PHA)	2	B	1	H3000759	56.79	13.59	0.00	0.00	70.38
2	1 (PHA)	2	B	8	H3000759	60.12	14.15	0.00	0.00	74.27
2	1 (PHA)	2	C	6	H3000760	54.01	12.86	0.00	0.00	66.87
2	1 (PHA)	2	C	7	H3000760	61.99	12.68	0.00	0.00	74.67
2	1 (PHA)	3	A	5	H3000764	1.75	0.50	0.00	0.00	2.25
2	1 (PHA)	3	A	6	H3000764	3.90	0.65	0.30	0.10	4.95
2	1 (PHA)	3	B	2	H3000765	4.79	0.74	0.72	0.30	6.55
2	1 (PHA)	3	B	3	H3000765	2.69	0.40	0.00	0.00	3.09
2	1 (PHA)	3	C	8	H3000766	3.19	0.82	0.00	0.00	4.01
2	1 (PHA)	3	C	9	H3000766	2.53	0.65	0.00	0.00	3.18
2	2 (LGC)	1	A	1	H3000770	6.72	2.37	0.00	0.00	9.09
2	2 (LGC)	1	A	3	H3000770	7.07	2.20	0.35	0.00	9.63
2	2 (LGC)	1	B	7	H3000771	9.58	2.96	0.00	0.00	12.54
2	2 (LGC)	1	B	9	H3000771	8.19	2.92	0.00	0.00	11.11
2	2 (LGC)	1	C	4	H3000772	7.39	2.57	0.00	0.00	9.96
2	2 (LGC)	1	C	5	H3000772	6.45	2.30	0.00	0.00	8.75
2	2 (LGC)	2	A	2	H3000776	11.36	1.88	5.23	1.55	20.02
2	2 (LGC)	2	A	4	H3000776	9.19	1.96	6.20	1.56	18.91
2	2 (LGC)	2	B	1	H3000777	8.62	1.76	6.97	1.52	18.87
2	2 (LGC)	2	B	8	H3000777	10.43	2.33	5.43	1.57	19.76
2	2 (LGC)	2	C	6	H3000778	9.17	1.80	5.20	1.27	17.45
2	2 (LGC)	2	C	7	H3000778	9.20	1.76	4.90	1.41	17.28
2	2 (LGC)	3	A	5	H3000782	56.05	9.36	0.00	0.00	65.41
2	2 (LGC)	3	A	6	H3000782	63.21	11.57	0.00	0.00	74.78
2	2 (LGC)	3	B	2	H3000783	48.66	6.65	0.00	0.00	55.31
2	2 (LGC)	3	B	3	H3000783	40.47	6.65	0.00	0.00	47.12
2	2 (LGC)	3	C	8	H3000784	32.25	5.72	0.00	0.00	37.98
2	2 (LGC)	3	C	9	H3000784	38.92	6.79	0.00	0.00	45.71
3	1 (PHA)	1	A	1	H3000943	21.11	2.23	0.00	0.00	23.34
3	1 (PHA)	1	A	3	H3000943	23.39	2.24	0.00	0.00	25.64
3	1 (PHA)	1	B	7	H3000944	20.16	2.19	0.00	0.00	22.35
3	1 (PHA)	1	B	9	H3000944	20.11	2.44	0.32	0.00	22.87

Lab	Consignment Number	10kg Sub-sample Number	Replicate Number	Run/ Batch	LIMS number	B1 (ng/g)	B2 (ng/g)	G1 (ng/g)	G2 (ng/g)	Total
3	1 (PHA)	1	C	4	H3000945	20.48	2.51	0.00	0.00	22.99
3	1 (PHA)	1	C	5	H3000945	18.74	2.14	0.00	0.00	20.88
3	1 (PHA)	2	A	2	H3000949	118.52	28.16	0.00	0.00	146.68
3	1 (PHA)	2	A	4	H3000949	87.74	27.94	0.00	0.00	115.68
3	1 (PHA)	2	B	1	H3000950	75.83	24.06	0.00	0.00	99.89
3	1 (PHA)	2	B	8	H3000950	74.28	23.62	0.00	0.00	97.90
3	1 (PHA)	2	C	6	H3000951	67.03	20.37	0.00	0.00	87.40
3	1 (PHA)	2	C	7	H3000951	64.31	17.12	0.00	0.00	81.44
3	1 (PHA)	3	A	5	H3000955	0.40	0.31	0.00	0.00	0.71
3	1 (PHA)	3	A	6	H3000955	1.45	0.28	0.00	0.00	1.73
3	1 (PHA)	3	B	2	H3000956	2.92	0.50	0.00	0.00	3.42
3	1 (PHA)	3	B	3	H3000956	1.34	0.15	0.00	0.00	1.49
3	1 (PHA)	3	C	8	H3000957	1.74	0.57	0.00	0.00	2.31
3	1 (PHA)	3	C	9	H3000957	1.21	0.40	0.00	0.00	1.61
3	2 (LGC)	1	A	1	H3000961	18.31	2.72	0.00	0.00	21.03
3	2 (LGC)	1	A	3	H3000961	18.74	2.56	0.00	0.00	21.29
3	2 (LGC)	1	B	7	H3000963	18.56	2.50	0.00	0.00	21.06
3	2 (LGC)	1	B	9	H3000963	19.33	3.01	0.00	0.00	22.34
3	2 (LGC)	1	C	4	H3000964	19.54	3.01	0.00	0.00	22.54
3	2 (LGC)	1	C	5	H3000964	17.64	2.42	0.00	0.00	20.06
3	2 (LGC)	2	A	2	H3000967	2.34	0.33	0.00	0.00	2.67
3	2 (LGC)	2	A	4	H3000967	1.01	0.22	0.00	0.00	1.23
3	2 (LGC)	2	B	1	H3000968	0.11	0.01	0.00	0.00	0.12
3	2 (LGC)	2	B	8	H3000968	1.14	0.37	0.00	0.00	1.51
3	2 (LGC)	2	C	6	H3000969	0.91	0.09	0.00	0.00	1.00
3	2 (LGC)	2	C	7	H3000969	1.11	0.29	0.00	0.00	1.41
3	2 (LGC)	3	A	5	H3000973	3.40	0.79	9.08	1.73	15.01
3	2 (LGC)	3	A	6	H3000973	4.23	0.77	5.00	1.20	11.21
3	2 (LGC)	3	B	2	H3000974	6.13	0.94	6.72	1.75	15.54
3	2 (LGC)	3	B	3	H3000974	4.61	0.80	7.72	1.78	14.91
3	2 (LGC)	3	C	8	H3000975	5.09	1.15	7.03	1.82	15.08
3	2 (LGC)	3	C	9	H3000975	4.25	0.98	7.43	1.74	14.40

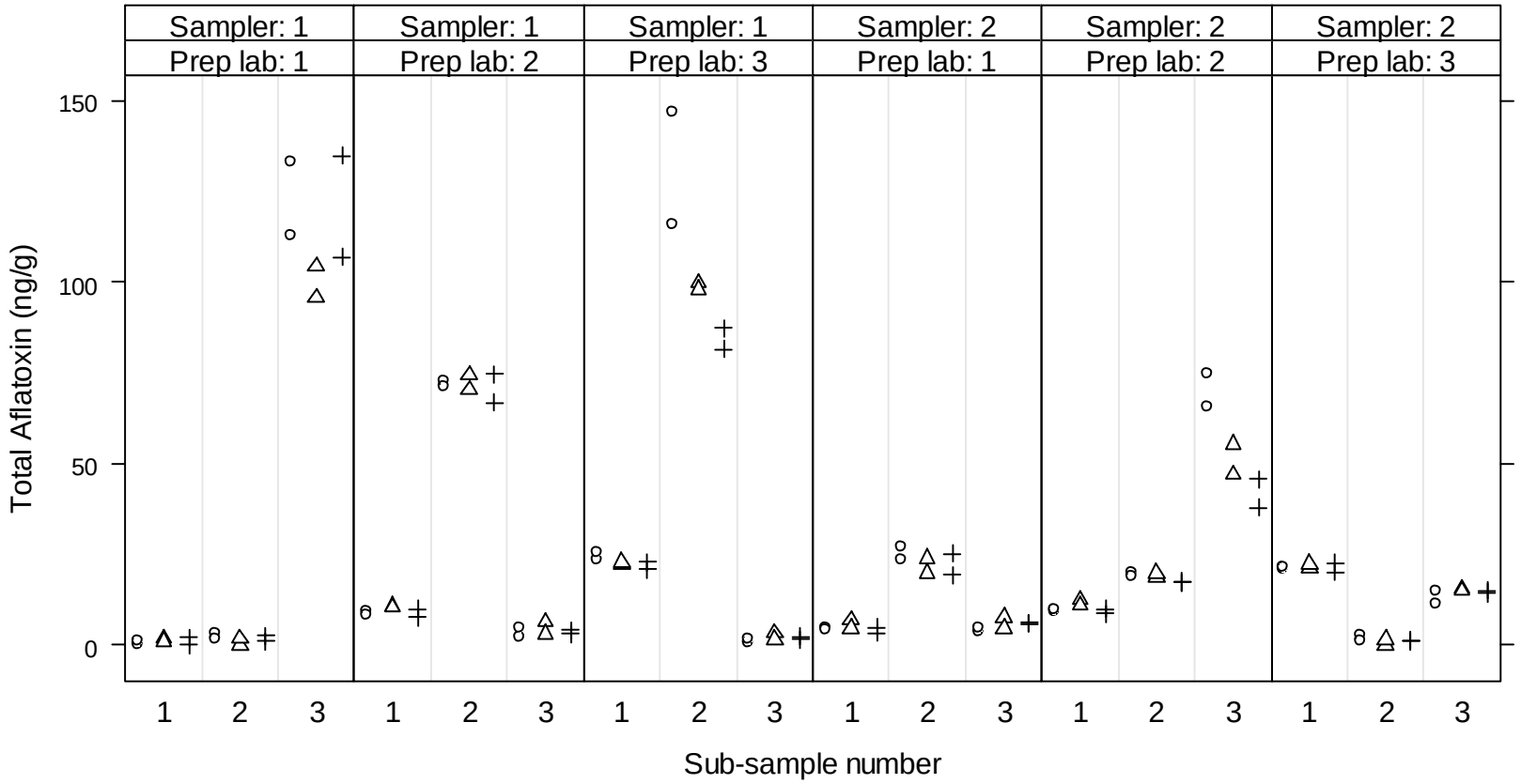
Figures 5 and 6 show the individual observations for aflatoxin B1 and total aflatoxin which indicate that agreement is generally good within a given sub-sample, but that different sub-samples from the bulk differ substantially. There is also a suggestion of different aflatoxin levels between the two primary sampling events; materials obtained by sampler 2 (GC) seem to have somewhat lower aflatoxin levels than those from sampler 1.

Figure 5: Summary of Aflatoxin B_1 Results



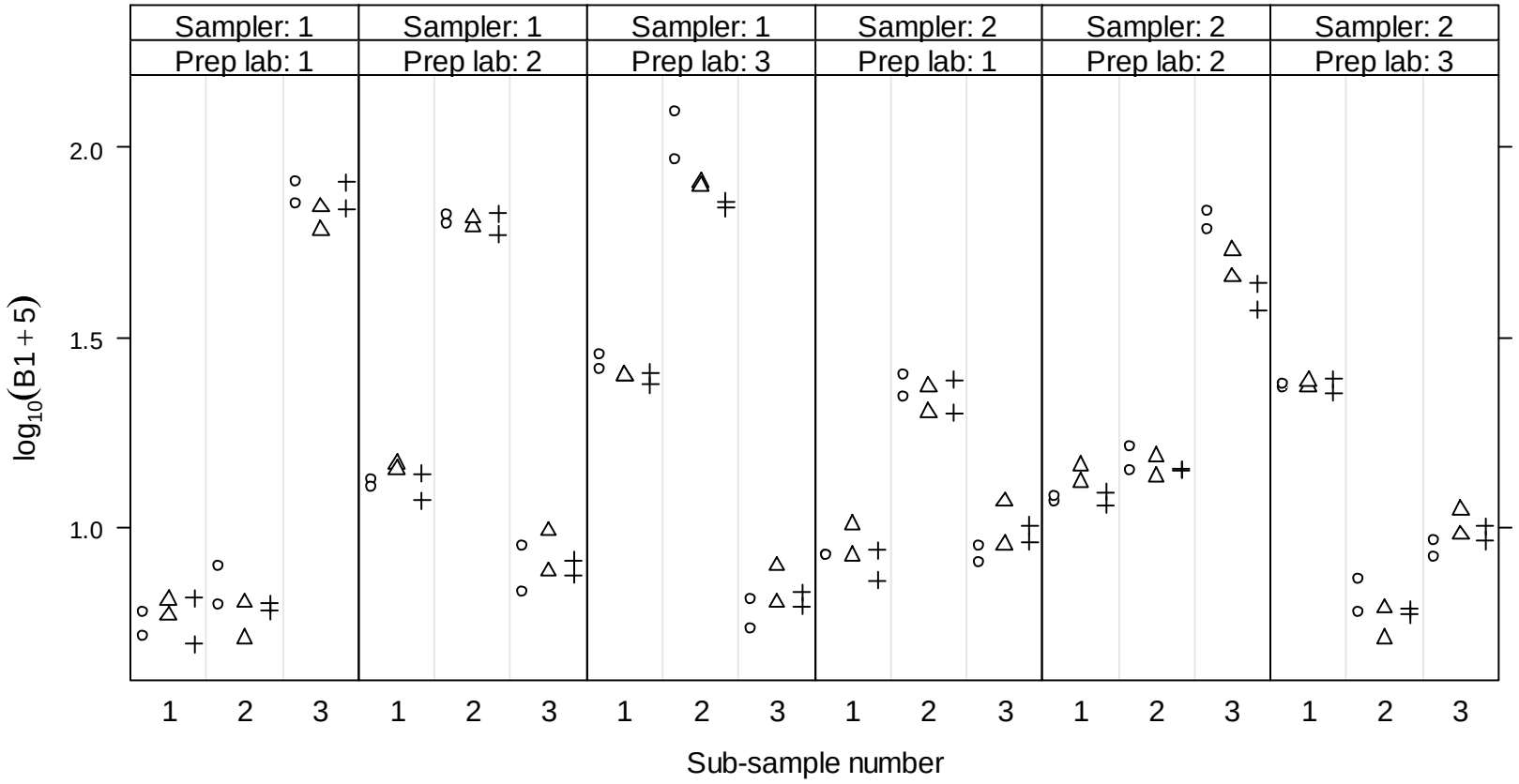
Aflatoxin B1 (recovery corrected), plotted by Sampler ("Sampler") and sample preparation lab ("Prep lab"). Symbols indicate the Replicate samples which would normally go to different laboratories, as follows: Circles (○): Prosecution lab (sample A); Crosses (+): Defence sample (Sample B); Triangles (Δ): Referee sample (Sample C).

Figure 6: Summary of Total Aflatoxin Results



Total Aflatoxin (recovery corrected), plotted by Sampler ("Sampler") and sample preparation lab ("Prep lab"). Symbols indicate the Replicate samples which would normally go to different laboratories, as follows: Circles (○): Prosecution lab (sample A); Crosses (+): Defence sample (Sample B); Triangles (Δ): Referee sample (Sample C).

Figure 7: Summary of Aflatoxin B_1 Results, Logarithmic Relationship



$\log_{10}$(Aflatoxin B1 + 5), plotted by Sampler ("Sampler") and sample preparation lab ("Prep lab"). Symbols indicate the Replicate samples which would normally go to different laboratories, as follows: Circles (○): Prosecution lab (sample A); Crosses (+): Defence sample (Sample B); Triangles (Δ): Referee sample (Sample C).

The statistical significance of the differences was assessed by analysis of variance, after a log-transformation to provide approximately homogeneous within-group variances and with duplicate observations averaged, analytical run effects having been found to be statistically significant at the 95% level. (The transformation used was y = log10(5+x), x being the raw aflatoxin result); Figure 7 shows the transformed data set for aflatoxin B1. Inspection of model residuals indicated reasonable homogeneity within-group and approximate normality of the residuals. The data structure is essentially a collection of two-way designs (corresponding to 10kg laboratory sample ID crossed with sample preparation laboratory), nested within an outer second two-way design (corresponding to sampler and aggregate 30kg sample). The effects present in this model are presented in Table 3.

Table 3 – Effects Present in the Statistical Model

Effect	Comment
1. The sampler	Between-sampler difference. Significance indicates a difference in the bulk sample content or the application of the sampling protocol.
2. Sample preparation laboratory	Differences between results for different sample prep labs. Significance indicates either different procedures or materially different 30 kg aggregate samples supplied to labs.
3. Sample preparation laboratory/sampler interaction	2-way interaction term (significance indicates that differences between sample prep labs depend on the bulk sample)
4. 10 kg laboratory sample*	Between-laboratory sample effects; significance indicates that different laboratory samples show materially different average aflatoxin content.
5. Replicate Test sample*	The replicate test sample label shows the grouping of these samples for regulatory purposes. Expected to arise only from random sampling effects.
6. Laboratory sample/replicate test sample interaction*	Interaction term for laboratory sample/replicate test sample (in real life sent to different testing laboratories). Significance might indicate significantly different laboratory sample portions.

* Effects 4-6 are nested within sampling lab/consignment

Regulatory maxima are set for aflatoxin B1 and for total aflatoxins. Aflatoxin B1 was found to be the dominant analyte and results for total aflatoxins followed similar patterns. Thus discussion centres on results for aflatoxin B1. A summary of the ANOVA results for aflatoxin B1 is given in Table 4. The most significant effect is, as expected from the Figures, the difference between 10kg laboratory samples, which is very strongly significant (p much less than 0.001). Within this, there is marginal evidence (p=0.07) of a difference between replicate test samples, and somewhat stronger evidence (p=0.02) of an interaction between Laboratory sample and replicate test sample, perhaps indicating some small differences

within the 10kg laboratory samples. The same general picture was observed for aflatoxin B2 and total aflatoxin levels. Note that the mean squares for the preparation laboratory and sampler effects were compared with the laboratory sample effect, implicitly treating the differences between 10kg laboratory samples as a random effect. The lack of statistical significance for sampler and sample preparation laboratory indicates that these are not important compared to the sampling variation found from one 10kg sub-sample to the next. The ANOVA results therefore support the existence of a very strong sampling effect causing differences between 10kg laboratory samples, but suggest that the apparent differences between samplers can be entirely accounted for by sampling variance.

Table 4 – ANOVA Results for Aflatoxin B1

Analysis of variance of log10(5+B1). F-statistics for consignment and sampling laboratory were calculated by comparing their mean squares to that for subsample.

Effect	***df***	***Sum sq***	***Mean sq***	**F**	**p-*value***
Sampler	*1*	*0.3602*	*0.3602*	*0.308*	*0.589*
Sample preparation laboratory	*2*	*0.6404*	*0.3202*	*0.274*	*0.765*
Sampler-sample preparation laboratory interaction	*2*	*0.6981*	*0.3490*	*0.298*	*0.748*
Laboratory sample	*12*	*14.0321*	*1.1693*	*588.1*	*<0.001*
Replicate Test sample	*12*	*0.0426*	*0.0035*	*1.785*	*0.074*
Laboratory sample- Replicate Test sample interaction	*24*	*0.0945*	*0.0039*	*1.981*	*0.019*
Residuals	*54*	*0.1074*	*0.0020*		

Although sampling effects appear to dominate the overall variance, the Laboratory sample/replicate test sample interaction suggests that measurement precision within a laboratory still includes some contribution from inhomogeneity (the whole (1kg) replicate test portion is not extracted for analysis). This, together with the tentative evidence of slurry ratio effects above, suggests that it may be useful to investigate means of improving the slurrying process further. More powerful homogenisation apparatus might be employed; however, speculating that aflatoxins may be being protected in the less wettable clumps of the mound they originate in, or by encapsulation in lipids from the groundnuts, it may be more economic to investigate the addition of a non-foaming surfactant to the slurry.

The observed distribution of aflatoxin content by 10kg laboratory sample is consistent with a situation in which approximately one in three of the 10kg laboratory samples contains hotspots of groundnuts with high levels of aflatoxins. The separation between these and the "background" laboratory samples (above 50ng/g or so compared to around 10ng/g) suggests that such laboratory samples probably contain only one or two hotspots and that 10kg is an appropriate size to detect aflatoxin contamination at this level. This corresponds with the findings of Cucullu *et al*, cited in reference 11 that most individual groundnuts have an aflatoxin concentration of zero but occasionally a groundnut may have an extremely high

aflatoxin concentration. In fact the distribution of variability is loaded predominantly onto sampling in relation to groundnuts and aflatoxins.

Conclusions

Measurement precision (within-replicate test portion variance) was generally good, though it deteriorates markedly for laboratory samples for which the aflatoxin content is very high. The between-replicate test portion standard deviation varied in a similar manner to the measurement precision.

The measurement precision still includes some contribution from inhomogeneity (the whole (1kg) replicate test portion is not extracted for analysis), and it is suggested that addition of a non-foaming surfactant to the slurry be investigated.

Most of the variance in the data was found to be at the 10kg laboratory sample level (between laboratory samples). This is consistent with previous findings[13]. However, at least one 10kg laboratory sample from each 30kg aggregate sample exceeded the statutory limit for Aflatoxin B1 (and total aflatoxins) in each of the six sampling exercises. Thus, measurements on what would have been the regulatory, defence and reference replicate test portions derived from laboratory samples in a "real" situation were always consistent with each other, indicating that sample homogenisation within laboratory samples is appropriate, and that replicate test samples sent to three separate measurement laboratories would be expected to produce very similar outcomes.

In summary, these results demonstrate that the EU-recommended sampling protocol and sample preparation procedures applied in UK enforcement laboratories are capable of being effective in assessing compliance with EU limits for aflatoxins in bulk consignments of in-shell groundnuts.

Acknowledgements

Funding is gratefully acknowledged from the UK Department for Business, Innovation & Skills (from July 2016, Department for Business, Energy & Industrial Strategy) as part of successive Government Chemist Programmes. Dave Pilbrow, Suffolk Coastal Port Health Authority, is thanked for sampling and access to the in-shell groundnuts used in the study. The following public analysts are thanked for carrying out sample preparation and allowing access to their laboratories: Peter Maynard – Kent Scientific Services, Shayne Dyer – Hampshire Scientific Services and Steven Guffogg – Lincolne, Sutton and Wood. The authors are grateful to Frank Torma, Bhavna Bhatt and Jesus Valcarcel for analysis carried out on the samples.

The views expressed in this paper are those of the authors alone. The paper should not be taken as an authoritative statement or interpretation of the law, as this is a matter for the courts. The authors are grateful to the Government Chemist for permission to publish this work.

References

1 D Benford, E Dogliotti, P Fürst, J-C Leblanc, O Lindtner, A Mutti, J Schlatter, JC Larsen and C Wild (2007), 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; EFSA J, **446**, 1–127, see p 86

2 J Nyikal *et al* (2004), Outbreak of Alfatoxin Poisoning Eastern and Central Provinces, Kenya, January–July 2004; Morbidity and Mortality Weekly Report, MMWR, Sept 3, 790-793 (Please see https://www.cdc.gov/mmwr/preview/mmwrhtml/mm5334a4.htm for full text and author list, accessed 21.11.2016)

3 C Probst, H Njapau and PJ Cotty (2007), Outbreak of an Acute Aflatoxicosis in Kenya in 2004: Identification of the Causal Agent; App Env Microbiol, **73**, 2762-2764

4 European Commission Reports of the Scientific Committee for Food (Thirty-fifth series) Luxembourg; Office for Official Publications of the European Communities 1996, see Opinion on Alfatoxins, Ochratoxin A and Patulin, p 45-50

5 Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting Maximum Levels for Certain Contaminants in Foodstuffs; See EUR-Lex http://eur-lex.europa.eu/homepage.html for latest amendments and consolidated version

6 The European Parliament and Council (2004) Regulation (EC) No 882/2004 on Official Controls performed to ensure the Verification of Compliance with Feed and Food Law, Animal Health and Animal Welfare Rules; Official Journal of the European Union, 28.5.2004, EN, L 191/1-L 191/52, See EUR-Lex http://eur-lex.europa.eu/homepage.html for latest amendments and consolidated version

7 TB Whitaker, AB Slate and AS Johansson (2005), Sampling Feeds for Mycotoxin Analysis; in The Mycotoxin Blue Book , Editor DE Durate, Nottingham University Press, Nottingham, England, p1-23

8 FG Giesbrecht and TB Whitaker (1998), Investigations of the Problems of Assessing Aflatoxin Levels in Peanuts; Biometrics, **54**, 739-753

9 TB Whitaker, WM Hagler Jr and FG Giesbrecht (1999), Performance of Sampling Plans to Detect Aflatoxin in Farmers' Stock Peanut Lots by Measuring Aflatoxin in High-risk-grade Components; JAOAC Int, **82**, 264-270

10 TB Whitaker, J Springer, PR Defize, WJ deKoe and R Coker (1995), Evaluation of Sampling Plans used in the United States, United Kingdom, and The Netherlands to Test Raw Shelled Peanuts for Aflatoxin; JAOAC Int, **78**, 1010-1018

11 TB Whitaker and AJ Johansson (2005), Sampling Uncertainties for the Detection of Chemical Agents in Complex Food Matrices; J Food Protection, **68**, 1306-1313

12 TB Whitaker (2006), Sampling Foods for Mycotoxins; Food Additives Contaminants, **23**, 50-61.

13 R Casado, M Parsons, DJ Weightman, RM Magan, N Origgi (2009), Modelling a Two-dimensional Spatial Distribution of Mycotoxin Concentration in Bulk Commodities to Design Effective and Efficient Sample Selection Strategies; Food Additives and Contaminants, **26**, 1298-1305

14 G Shephard, F Berthiller, J Dorner, R Krska, G Lombaert, B Malone, C Maragos, M Sabino, M Solfrizzo, M Trucksess and H Van Egmond (2010), Developments in Mycotoxin Analysis: an Update for 2008-2009; World Mycotoxin J, **3**, 3-23

15 F Berthiller, C Brera, C Crews, MH Iha, R Krsha, VMT Lattanzio, S MacDonald, RJ Malone, C Maragos, M Solfrizzo and J Stroka (2015), Developments in Mycotoxin Analysis: an Update for 2013-2014; World Mycotoxin J, **8**, 5-35

16 TB Whitaker, AB Slate, MB Doko, BM Maestroni and A Cannavan (2010), Sampling Procedures to Detect Mycotoxins in Agricultural Commodities; Springer, Dorndrecht

17 M Thompson and T Fearn (1996), What Exactly is Fitness for Purpose in Analytical Measurement?; Analyst, **121**, 275-278

18 MH Ramsey, J Lyn and R Wood (2001), Optimised Uncertainty at Minimum Overall Cost to Achieve Fitness for Purpose in Food Analysis; Analyst, **126**, 1777-1783

19 T Fearn, SA Fisher, M Thompson and SLR. Ellison (2002), A Decision Theory Approach to Fitness for Purpose in Analytical Measurement; Analyst, **127**, 818-824

20 TB Whitaker (2003), Standardisation of Mycotoxin Sampling Procedures: an Urgent Necessity; Food Control, **14**, 233-237

21 Commission Regulation (EC) No 401/2006 of 23 February 2006 Laying down the Methods of Sampling and Analysis for the Official Control of the Levels of Mycotoxins in Foodstuffs; Official Journal of the European Union L 70/12, 9.3

22 Guidance Document for Competent Authorities for the Control of Compliance with EU Legislation on Aflatoxins;

https://ec.europa.eu/food/sites/food/files/safety/docs/cs_contaminants_sampling_analysis-guidance-2010_en.pdf (accessed 17.12.2016)

23 https://www.gov.uk/government/organisations/government-chemist

24 Office of Public Sector Information (UK), Food Safety Act 1990

25 Office of Public Sector Information (UK), The Food Safety (Sampling and Qualifications) Regulations, 2013; (made separately in each country of the UK)

26 J Velasco and SL Morris (1976), Use of Water Slurries in Aflatoxin Analysis; J Agric Food Chem, **24**, 86-88

27 European Committee for Standardisation, CEN, (2006), Foodstuffs - Sample Comminution for Mycotoxins Analysis – Comparison between Dry Milling and Slurry Mixing; PD CEN/TR 15298:2006. Available from BSI

28 TB Whitaker, JW Dickens, and RJ Monroe (1980), A Water Slurry Method of Extracting Aflatoxin from Peanuts; J American Oil Chemists Soc, **57**, 269-272

29 MC Spanjer, JM Scholten, S Kastrup, U Jörissen, TF Schatzki and N Toyofuku (2006), Sample Comminution for Mycotoxin Analysis: Dry Milling or Slurry Mixing?; Food Additives Contaminants A, **23**, 73-83

30 R-Biopharm Ltd 2005, Kit Insert for Easi-Extract® Aflatoxin, Product Code P71/RP70NEE Aflatoxin IFU (RPv8).doc20.10.16

31 R Development Core Team (2008), R: A Language and Environment for Statistical Computing; R Foundation for Statistical Computing, Vienna, Austria; ISBN 3-900051-07-0

32 R - URL http://www.R-project.org