The Food and Environment Research Agency
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Report FD 12/01 -

FSA Project FS241008 -

Study to Compare the Aflatoxin Content of Brazil Nut Kernels with that of the Shell

PROJECT INFORMATION

Report title:	Study to Compare the Aflatoxin Content of Brazil Nut Kernels with that of the Shell
Fera Report No.:	FD 12/01
Fera Project No.:	V7XV
FSA Project No.:	FS241008
Report Status:	Final
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Report approved by:	Castle, L.
Date:	January 2012

EXECUTIVE SUMMARY

Brazil nuts are a crop that can frequently be contaminated with aflatoxins.Limits are in place to control the concentration of aflatoxins and these assume the aflatoxin is in the edible portion of the nuts. Because any contamination can be very unevenly distributed, a large sample size of typically 20 kg of nuts is recommended to ensure a representative sample for testing. Such a large sample is difficult, time-consuming and expensive to shell manually. Consequently, current EU Regulations permit compliance to be checked by measuring the aflatoxin concentration in the in-shell nuts and applying a conversion factor of approximately 2 to convert the measured concentration to an estimate of the concentration in the edible portion. This conversion factor of 2 is based on the supposition that all aflatoxin measured in the in-shell nuts is present in the edible portion - the kernel - and that the kernel and shell are of approximately equal mass. The aim of this project was to examine results of the measurement of aflatoxin in the shells and kernels of Brazil nuts to establish the relationship between the concentration in whole nuts (the concentration usually measured) and in the kernel (the concentration of interest); i.e. to determine whether or not the conversion factor described above is appropriate. To achieve this, the project was set up in two stages. In the first part the aim was to collect and collate existing data from a variety of sources and to use this data to try to develop a statistical model or conversion factor that describes the relationship between aflatoxin contamination of Brazil nut kernels and the corresponding shells. In the second part the aim was to test this model or conversion factor using additional data generated in this project (by the analysis of whole in-shell nut samples, separated into kernel only samples and shell only samples).

Part 1 – The most comprehensive data set received was provided by Vargas *et al*. and was published in 2011. This data was from the Brazilian 'CONFORCAST' project. The data considered was derived from the analysis of twelve lots each separated into ten samples consisting of at least 30 kg of un-sorted in-shell nuts. Ten samples from five of the lots and one sample from each of the other seven lots were selected for shelling and analysis. Some samples were incomplete, so in total data from 54 samples (minimum 30 kg each) were considered. The samples were separated into several fractions with data being provided for: good kernels, good shells, rotten kernels and rotten shells. Our assessment of the data provided concluded:

- There was no clear relation between the concentration of aflatoxins and the proportion of aflatoxins in the kernel in 30 kg un-sorted samples taken from the lots.
- The proportion of aflatoxin in kernels was, on average, lower in sorted samples (rotten nuts removed) than in un-sorted samples.
- A mean (across-lot) conversion factor of 1.05 was estimated from the CONFORCAST data (with a 95% confidence interval of 0.86 to 1.25 on the mean), i.e. lower than the currently applied conversion factor of approximately 2. A conversion factor of 1 means that the aflatoxin concentration is on average the same in the kernel as in the shell therefore a

concentration measured for in-shell nuts gives directly, without change, an estimate of concentration in the kernels.

- The analytical measurement and the conversion factor each have an associated uncertainty. Where a measurement is used to estimate the concentration of aflatoxins in the kernels of nuts in a particular lot the analytical uncertainty is typically estimated to be ± 44% (based on Horwitz). The results examined in this study suggest that the uncertainty associated with the estimated concentration of aflatoxins in the kernel of a lot based on an in-shell measurement result converted to a kernel concentration using a factor of 1 is ± 80%. The main source of additional uncertainty being between-sample variation in the proportion of aflatoxins in the kernel and on the shell.

- Current practice for assessment against the legislative limit is to measure aflatoxins in in-shell samples, then correct this for analytical recovery. The analytical measurement uncertainty associated with the result is estimated. It is assumed that all measured aflatoxins are in the kernel and a conversion factor of approximately 2 (with no associated uncertainty) is applied, which in effect doubles the concentration and its associated uncertainty. Then if the converted result is unequivocally above the limit when the converted analytical uncertainty is taken into account the sample is declared to be non-compliant. The results of this study show that this is likely to lead to estimates of concentration in the kernel which are biased upwards and with an estimated uncertainty that is too small. For example, this study shows that an in-shell result of 9 µg/kg is consistent with a kernel concentration of 1.8 to 16.2 µg/kg (conversion factor of 1 and combined uncertainty of ± 80%), i.e. non-compliance is not demonstrated. However under current practice the kernel concentration would be reported as 18 ± 7.9 µg/kg (conversion factor of 2 and analytical uncertainty of ± 44%), a result that demonstrates non-compliance.

- Where in-shell measurements are used to estimate the concentration of aflatoxins in kernels, the results examined in this study show that variation in proportion of aflatoxins in the kernel and shell means that a result of approximately 50 µg/kg in-shell is needed in order to demonstrate that kernels are non-compliant at a level of 10 µg/kg (correction factor of 1 and combined uncertainty of ± 80%).

- A third option is to measure the concentration of aflatoxins in shelled kernels. This reduces the expected uncertainty about the concentration of aflatoxins in kernels from ± 80% to ± 44% at the cost of increased sample preparation. Here a result of 18 µg/kg can be expected to demonstrate non-compliance.

- The currently applied conversion factor of approximately 2 that presupposes that all aflatoxin occurs in the kernel estimates the worst case for consumption, and so in effect offers the highest consumer protection, with the associated risk that compliant lots may be rejected.

Part 2 – Eleven lots each consisting of 2 kg of un-sorted in-shell nuts were shelled and tested at Fera. Each sample was separated into three fractions: kernels, shells with kernel residue and shells

without kernel residue, prior to analysis. Differences in sample mass and the fact that all of the nuts were visibly rotten, and so were not generally representative of typical batches of edible nuts, meant that the value of the conversion factor calculated in Part 1, could not be validated or refuted by results generated by the analysis of these independently sourced Brazil nuts.

In conclusion, based on the CONFORCAST results a conversion factor of 1 seems applicable to an in-shell aflatoxin concentration range of 4 to 100 µg/kg in 30 kg samples. However further work is required to fully validate this for use for smaller samples in the range 2 – 12 kg (as currently specified in Commission Regulation (EU) No. 178/2010). For concentrations of > 100 µg/kg in the nuts the conversion factor becomes irrelevant as the nuts are clearly non-compliant with the limits for direct human consumption (10 µg/kg) or for product to be subjected to further processing (15 µg/kg).

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ABBREVIATIONS & DEFINITIONS

ABBREVIATIONS -

AFB1	Aflatoxin B_1 -
AFB2	Aflatoxin B_2 -
AFG1	Aflatoxin G_1 -
AFG2	Aflatoxin G_2 -
HPLC	High Performance Liquid Chromatography -
LC-MS/MS	Liquid Chromatography Mass Spectrometry/Mass Spectrometry -
QA/QC	Quality Assurance /Quality Control -
UKAS	United Kingdom Accreditation Service -

DEFINITIONS -

For the CONFORCAST project the following definitions were used (as given in Vargas *et al.*, 2011): -

Fractions: Any part of Brazil nut derived from the shelling and sorting -

Rotten kernels: nuts easily segregated visually by a consumer (empty, mouldy, fermented, cut, - rotten or black) -

Rotten shells: shells from rotten kernels -

Good kernels: kernels with no visible damage not rejected by consumers -

Good shells with kernel residue: shells from good kernels that had part of the kernel attached -

Good shells without kernel residue: shells from good kernels that had no residue attached -

Rotten nuts: mass balance of rotten kernels and rotten shells -

1. INTRODUCTION

The UK market for Brazil nuts in-shell is high compared to the rest of the EU, importing over 500 tonnes of Brazil nuts in-shell in 2009, (website: http://exporthelp.europa.eu). Brazil nuts are a crop that can frequently be contaminated with aflatoxins. Aflatoxins, and in particular aflatoxin B_1, are considered to be genotoxic and carcinogenic and there is evidence they can cause liver cancer in humans (FAO/WHO, 1998). Limits have been set and recently revised by the European Commission (Commission Regulation (EU) No. 165/2010) to limit aflatoxins in ready to eat Brazil nuts to 5 µg/kg aflatoxin B_1 and 10 µg/kg total aflatoxin. For Brazil nuts not ready to eat but that will be subject to further sorting the respective limits are 8 µg/kg aflatoxin B_1 and 15 µg/kg total aflatoxin. This brings the regulated levels into line with limits of total aflatoxin of 10 µg/kg for ready to eat Brazil nuts and 15 µg/kg for Brazil nuts destined for further processing agreed at Codex (Codex, July 2010).

Because any contamination can be very unevenly distributed, a large sample size of typically 20 kg of Brazil nuts is recommended to obtain a representative sample for testing. Such a large sample is difficult, time-consuming and expensive to shell manually. Laboratories do however have equipment that can grind the whole nuts to a small particle size suitable for analysis. Consequently, current EU Regulations permit compliance to be checked by measuring the aflatoxin concentration in the whole nut (Commission Regulation (EC) No 401/2006), and applying a conversion factor of approximately 2 to increase the measured concentration to an estimate of the concentration in the edible portion. However Regulation (EU) No. 165/2010 also contains the footnote:

'The maximum levels refer to the edible part of groundnuts (peanuts) and tree nuts. If groundnuts (peanuts) and tree nuts "in shell" are analysed, it is assumed when calculating the aflatoxin content all the contamination is on the edible part, except in the case of Brazil nuts'.

Although this statement is included in the legislation there is no recommendation as to how to treat the data derived for Brazil nut samples analysed in-shell. It is unclear to what proportions aflatoxin contamination occurs in the kernel and on the shell of the Brazil nut. The Food Standards Agency has asked for information to help understand the distribution of contamination between the kernel and the shell to inform setting limits and sampling protocols.

2. PROJECT OBJECTIVES

The project was broken down into the following objectives:

Objective 1. Collate information about aflatoxin distribution in Brazil nut kernels and shells, using sources from published literature and unpublished data, and to use this data to derive the relationship between whole nut aflatoxin levels and kernel aflatoxin levels

Objective 2. Source samples for analysis

Objective 3. Assessment of nut-shelling process

Objective 4. Hand shelling and sample preparation

Objective 5. Sample analysis for aflatoxins

Objective 6. Validate / test model using data from Objective 5

Objective 7. Final report

The objectives were addressed in two parts. In the first part (Objective 1) the stated aim was data would be collected and collated from a variety of sources (published and unpublished). Any data provided would be used to try to develop a statistical model or conversion factor that describes the relationship between aflatoxin contamination on Brazil nut kernels and the corresponding shells. The second part of the project was to address Objectives 2 to 7. Data from analysis of samples obtained by Fera would be used to validate the model. A stop-go decision was included after Objective 1 in case there was already a large amount of data available of suitable quality and so no further analysis would be required. In the event both Part 1 and Part 2 of the project were conducted.

3. MATERIALS AND METHODS

Objective 1. Collate information about aflatoxin distribution in Brazil nut kernels and shells, using sources from published literature and unpublished data.

A wide range of sources were used to identify information and data that could be used in the production of a statistical model or factor that could be used to describe the distribution of aflatoxin between Brazil nut kernels and shells.

Published Literature **–** Literature searches were carried out using several online search facilities. Internet sources searched included: Web of Knowledge, Science Direct, Ovid Online and Swetswise. Searches were conducted using key words in different combinations, key words used included: aflatoxin(s); Brazil; Brazil nut(s); shell(s); kernel(s). Initially searches were carried out over the period since 2000 as it was thought that there might be a lot of information and that the quality of any analytical data would be better in more recent publications. As this search gave only limited returns the search was widened to cover the period going back to 1965. The searches were carried out early in 2011 and further update searches were carried out in July 2011.

Specifically the data reported as part of the Swedish NFA project (Marklinder *et al*., 2005), data from SAFENUT project (STDF project 114 Final Report), data from INC and from the Brazilian Codex Submission on Brazil nuts (CAC, 2010) was considered.

Unpublished data **–** A number of individuals and organisations active in aflatoxin analysis were contacted and asked if they had data they could provide.

Data evaluation and production of an initial model **–** The aim of the data analysis was to find a relationship between the concentration of aflatoxin in whole nuts (the value usually measured) and the concentration of aflatoxins in kernels (the value of interest). Statistical evaluation of the data obtained in Objective 1 was carried out. A summary of the statistical approach is given in Appendix 2.

Objective 2. Source samples for analysis

Extensive efforts were made to secure samples for this project during the proposal writing and project negotiation stages. Further efforts were made once the project started. A number of individuals, institutes, laboratories and organisations including retailers and Trade Associations were contacted by email to request assistance in obtaining samples.

Objective 3. Assessment of nut-shelling process

Pre-trials to assess the most efficient way to cleanly shell nuts were carried out. On-line information suggested freezing the nuts prior to shelling can help by making the shell brittle and separating the kernel from the shell so it can be removed cleanly. This is suggested for domestic hand shelling.

Three types of nut cracker were tested.

1. A traditional squeeze type, with one pinch point that grips and cracks the nuts.
2. A traditional squeeze type, with two pinch points.
3. 'Top Cracker' – a cup shaped holder that squeezes to crack the nuts.

Shelling was assessed for nuts from 2 different batches that had been stored at 3 different temperatures; room temperature (~21°C) and frozen (-18°C and -80°C).

Objective 4. Hand shelling and sample preparation

Hand shelling – All samples sourced in Objective 3 were hand shelled using cracker 3. This was carried out in a fume cupboard as many of the nuts were spoiled and smelt very strongly rancid or 'bad'.

Sample preparation and homogenisation (kernels) – Frozen kernel samples were allowed to reach room temperature before preparation. Kernels were homogenised with water to form a slurry using a laboratory MagiMix blender. Samples were roughly ground initially, and then portions of tap water added to produce a smooth slurry. The amount of water added varied but was usually in the ratio of equal parts of water : kernel, the exact amount was recorded for each sample, and was dependant on how well the sample mixed. Each sample was mixed with the final water volume for 30 minutes. Sub-sample aliquots (~200 g) were transferred to plastic sample pots to produce laboratory samples, the remainder was transferred to plastic sample bags for storage. All samples were stored in a freezer until analysis.

Sample preparation and homogenisation (shells) – Shell samples were milled using a Retsch centrifugal mill fitted with a 1 mm screen. The samples were milled until a fine powder was obtained. In some cases it was necessary to pass the sample through the mill twice to ensure the particle size was small enough. After milling, samples were mixed in a Pascal Tumble mixer for 30 minutes, then stored in a freezer until analysis.

Objective 5. Sample analysis for aflatoxins

Kernel samples – Kernel samples were extracted using a UKAS accredited method for aflatoxins in nuts. Slurried sample equivalent to 20 g kernel was weighed into a beaker. Actual weights were recorded and were in the range 36 - 45 g. Acetonitrile and water were added to make the final ratio equivalent to 60:40, v/v, including the water used in the slurry process. This was done to ensure consistency of results and to make final calculations more straightforward. Samples were extracted by blending using an Ultra Turrax homogeniser for 3 - 5 minutes. The extract was filtered and an aliquot diluted with Phosphate Buffered Saline (PBS).

Shell samples – Shell samples (20 g) were weighed into round bottom flasks to which acetonitrile : water (60:40, v/v) was added. The flasks were sealed and placed on a wrist action shaker for 2 hours. After extraction the samples were filtered through Whatman 113V (equivalent) filter paper and an aliquot was diluted with PBS.

In both cases the diluted filtrate was cleaned-up on immunoaffinity column (Easi-Extract, R-Biopharm Rhone) by an automated system (Gilson, ASPEC), then analysed by HPLC with fluorescence detection and post column derivatisation (KOBRA cell) (Sharman and Gilbert, 1991). In-house reference samples (previous FAPAS materials – a nut based animal feed and Brazil nut slurry) and spiked samples were analysed with the test samples. All samples were analysed in duplicate. Any samples found to be outside the calibration range (0-10 µg/kg) were diluted and reanalysed to ensure an accurate quantification of the aflatoxin levels.

Objective 6. Validate / test model using data from Objective 5

The results of the aflatoxin analysis of the Fera obtained samples were considered. Further information is provided in Section 4. RESULTS AND DISCUSSION.

4. RESULTS AND DISCUSSION

Objective 1 – Collate information about aflatoxin distribution in Brazil nut kernels and shells, using sources from published literature and unpublished data

Published Literature – When literature searches were carried out using only the term ‘aflatoxin’ tens of thousands of results were found, however using any combination of that with ‘Brazil nut’ or ‘shell’ or ‘kernel’ returned relatively few hits. The majority of these dealt with mycology, i.e. isolation and identifying species of mould on the nuts, or different thresholds for water activity/moisture etc. for aflatoxin formation, or were simply reports of aflatoxin levels in Brazil kernels (Arrus *et al*., 2005a, Arrus *et al*., 2005b, and Freire *et al*., 2000). A paper by Johnnson *et al*. (2008) described inoculation experiments carried out to gain more information about the effect of temperature, humidity and storage time on aflatoxin development. This work was part of the SAFENUT project (STDF project 114 Final Report).

The SAFENUT project, short title “Prevention and control of aflatoxins in Brazil Nuts” was funded by the Standards and Trade Development Facility in Brazil. The project had five broad objectives

including; characterisation of the Brazil nut production chain; validation of recommended good practices……for aflatoxin control; validation & implementation of a rapid surveillance system, and knowledge transfer. The project was focussed on identifying key points where contamination could occur and providing solutions to prevent, control & monitor aflatoxins, in addition to providing training on rapid test kits to personnel involved in the Brazil nut supply chain. Further details including the final report (STDF project 114 Final Report) are available from the project website : http://www.stdf-safenutproject.com/. The project did not address the question of aflatoxin distribution between shell and kernel and as such did not provide any data for use in the development of a model/conversion factor.

Marklinder *et al.* (2005) conducted a study to determine consumers' ability to discriminate aflatoxin contaminated Brazil nuts. Consumers were asked to crack Brazil nuts and sort them into those they would consider edible and those they would consider inedible. Both 'edible' and 'inedible' kernel portions were analysed for aflatoxin. Based on the data the authors concluded that consumers can discriminate contaminated nuts to a certain extent, based on the physical appearance of the kernel after shelling. During the study, a small number of shell samples from samples chosen as edible and inedible, covering a range of aflatoxin levels were selected for aflatoxin analysis. In a personal communication Dr Monica Olsen observed that at low levels the distribution is about 50/50, (kernel/shell) but at higher levels the aflatoxin level is much higher in the kernel. Although this study addressed aflatoxin distribution the data could not be used for statistical analysis as the sample size (300 g) was too small.

One paper discussed the relationship between selenium and aflatoxin levels in Brazil nuts from the Amazon Basin (Pacheco and Scussel, 2007). After culturing surface sterilised nuts, it was noted that Brazil nuts had much higher levels of internal colonisation of moulds than pistachios, almonds and walnuts (Bayman *et al*., 2002). The authors suggested that as the nuts are collected in the wild and stored and transported under conditions that could favour fungal growth, fungi have more opportunities to colonise internal tissues than in other nuts. They reasoned that this would support the hypothesis that the mould and therefore the aflatoxin is found mainly in the kernel, however no aflatoxin analysis was carried out to prove this.

There were also papers and reports describing various means to sort Brazil nuts to remove contaminated nuts from lots, using size, colour and/or fluorescence. One recent paper by Pacheco and Scussel (2009) states 'There is not much published data on aflatoxin levels in Brazil nuts, only sporadic information published as reports and some aflatoxin data does not specify the origin of the Brazil nut samples…'. In their publication they describe an LC-MS/MS method for aflatoxin analysis of Brazil nuts, and they report the results of aflatoxin analysis of 171 samples of Brazil nuts. The nuts were from two harvest years, both in-shell and shelled nuts were tested and these were also graded according to size. They discussed how storage conditions, moisture content and water activity may affect aflatoxin levels and noted a difference between harvest years; fewer nuts from 2007 were found to contain aflatoxin and at lower levels than samples from 2006. They also discussed how samples could be better sorted before sale to remove or reduce the number of discoloured or bad nuts. They did not make any assessment of the contribution of the shell to any aflatoxin levels. Furthermore it was stated that before analysis in-shell nuts were shelled and all kernels (good and bad) included in analytical sample. This means any aflatoxin measured in their study was from the kernel only.

Steiner *et al*. (1992) assessed fluorescence and aflatoxins in Brazil nuts and pistachio nuts. They noted that not all fluorescent kernels were contaminated with aflatoxins, and brown spotted kernels also contained the toxins. They tested individual pistachio kernels and shells and showed comparatively small amounts of aflatoxin in the shell. Aflatoxins were only found in shells from kernels that contained 1400 mg/kg and 218 mg/kg respectively. Other highly contaminated pistachio kernels (up to 18 mg/kg) did not have aflatoxin present in the shells. They were also able to estimate the ratio of uncontaminated to highly contaminated pistachio nuts as 4700 to 1 and 4300 to 1 for the two pistachio nut samples from the lots tested. A similar ratio could not be calculated for Brazil nuts as not all highly contaminated kernels were analysed individually. The paper noted that occurrence of contaminated Brazil nuts was more frequent but at a lower (less extreme) level than in pistachio nuts. The highest aflatoxin level found in one Brazil nut kernel was 4 mg/kg.

DeMello and Scussel (2007) described various characteristics of in-shell Brazil nuts, and grouped them into three groups according to their overall length, weight and shell chromaticity. They also established a shell/nut factor (*F*) and considered that an F-factor of 1.5 (i.e. 60% shell, 40% kernel) was the boundary ratio for normal healthy whole Brazil nuts. According to this measure, whole nuts with $F < 1.5$ were considered healthy and $F > 1.5$ were considered deteriorated. The ratio is increased (i.e. proportionately more shell) in deteriorated nuts due to a number of factors that lead to loss of weight of the edible kernel, including loss of moisture, and reduction of the mass of the edible part by fungi. In this study aflatoxin measurements were only made on kernels, shells were not tested for aflatoxin.

Two articles by the same authors report the use of ozone during Brazil nut storage to reduce fungal contamination and also aflatoxin levels in in-shell Brazil nuts (Giordano *et al*., 2010 and Scussel *et al*., 2011). The other new article found was a review discussing the relative health benefits and risks associated with consumption of Brazil nuts (Freitas-Silva and Venâncio, 2011). None of these articles contained any new or relevant information about the proportion of aflatoxin contamination in Brazil nut shells versus the kernels.

As well as the searches of literature databases, extensive searches were made of the internet using Google and other search facilities. While many hits were produced, the scientific papers were those already found by searching literature. The information found was varied including; various reports of the dangers of consuming Brazil nuts because of aflatoxins; discussions and news items about the lack of availability of Brazil nuts due to controls; on-line sales and various commercial offers; raw food pages and blogs etc.; European Regulation information and various other items relating to Brazil nuts. A report by de Mello and Scussel (2009) about the development of sorting equipment was also found, but again no relevant data on aflatoxin levels in shells were included.

In summary, there was no useful or relevant data available in the published scientific literature that could be used for this study, apart from the results of the shell and kernel analysis from Marklinder *et al*. (2005). This data was limited in its use due to the very small sample sizes (~300 g) used in the study, and therefore it was not used in the statistical modelling.

Unpublished data **–** Very few of the laboratories contacted received Brazil nut samples to test routinely. Those that had carried out analysis used methodology that did not permit the analysis of the separate components (i.e. shell and kernel) and therefore could not provide data for this project.

The INC Scientific Committee provided the final report of the SAFENUT project (available from the website: STDF project 114 Final Report). Subsequently they also provided unpublished data from laboratory analysis results from industry sources concerning aflatoxin performed on export lots, as well as data from large importers. This data comprised of results collected in 2003 and 2006 for consignments or samples of Brazil nuts. The data was a mixture of results for whole nuts and shells and kernels for samples tested in Brazil and Italy. It was not used in the development of the model/conversion factor as no information on sample size, method of analysis, quality assurance parameters and whether or not the data was recovery corrected could be obtained. Interestingly this data also showed a pattern of low levels of aflatoxin present on shells when very low level or no aflatoxin was present in the kernels, however in the samples that exceeded the maximum limit the aflatoxin concentration in the kernel was approximately double that of the shell, i.e. in agreement with the personal communication with Dr Monica Olsen described above.

The Brazilian CONFORCAST project involved the determination of the aflatoxin content of kernels and shells of good and rotten Brazil nuts. Although publicly available through the Brazilian Codex submission on draft maximum levels for aflatoxins (CAC, 2010) the data obtained in the CONFORCAST project was not presented in sufficient detail to allow its use in the model development as individual data points were required. A confidentiality agreement was signed and the data (with supporting information on sampling, recovery and quality assurance for the results) was provided by Dr Vargas, LANAGRO/MG MAPA, Brazil. Within the timeframe of this project the results of the CONFORCAST project were published in the scientific literature (Vargas *et al*., 2011). An overview of the work carried out and the measurement results provided are given in Appendix 1. Further information about the CONFORCAST project can also be found in Codex Documents (CAC 2009a, 2009b, 2009c and 2010). This data contained the information required and so was used in the statistical work for this study. Of all of the data received and considered only the CONFORCAST data included sufficient numbers of replicates of sufficient mass; information about how samples were taken; information about sample sizes, and analytical quality assurance that allowed data to be used.

Data evaluation and production of an initial model

Inspection of results – Inspection of the concentrations of aflatoxins in kernels, shells and whole nuts generated in the CONFORCAST project showed that there was an apparent approximate linear relation between the concentration of aflatoxins in shells and kernels with the exception of a single sample which produced a high-leverage outlying point with respect to the remaining data. A regression with the point removed showed a linear relation with an intercept close to zero (not shown). Similar correlations are shown in Vargas et al. (2011), in some cases with data removed. It was not considered acceptable to remove any data on the basis of their value. However, it was not possible to use results expressed as a concentration because of the presence of that point. Expressing results as proportions enabled all data to be analysed.

Relation between concentration of aflatoxins in whole nuts and the proportion of aflatoxins in the kernel – The aim was to describe the relation between proportion of total aflatoxins in the kernel - and easily observable (in the context of control) quantities, such as measured concentration of -

aflatoxins in whole nuts and the occurrence of rotten nuts, which could then be used to describe the relation between the concentration of aflatoxins in whole nuts and in kernels.

Linear regression of log transformed concentration in nuts against the proportion in the kernel was undertaken for all nuts, rotten nuts and good nuts. Similarly the relation between log transformed concentration and the proportion of rotten nuts in the sample was examined. A regression of the concentrations of aflatoxins in good and rotten nut samples (based on visual inspection described in the CONFORCAST project, see Definitions & Appendix 1) showed that the concentration of aflatoxins in good nuts, which contained aflatoxins at concentrations up to approximately 32 µg/kg, tended to increase as the proportion of aflatoxin in the kernel increased (Figure 1), whereas the concentration of aflatoxins in samples of rotten nuts, which contained aflatoxins at concentrations between approximately 100 and 1500 µg/kg, tended to decrease as the proportion of aflatoxin in the kernel increased (Figure 2). The between-sample variation was large within both of these relations. This means that these relations may not be very useful for the production of a more nuanced model. The combined effect of these relationships for 'real' (un-sorted whole) samples containing both good and rotten nuts was that the concentration of aflatoxins was not related to the proportion of aflatoxin in the kernel (Figure 3 and Appendix 2).

Figure 1: Relation between concentration of aflatoxins in nuts and proportion of aflatoxins in the kernel (good nuts)

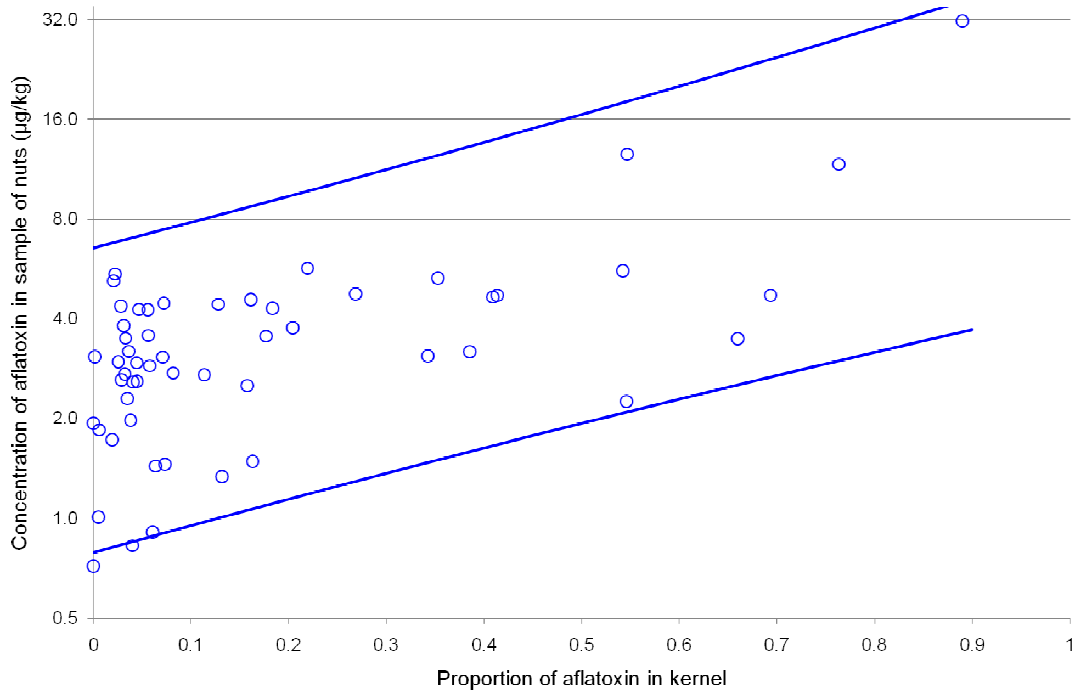
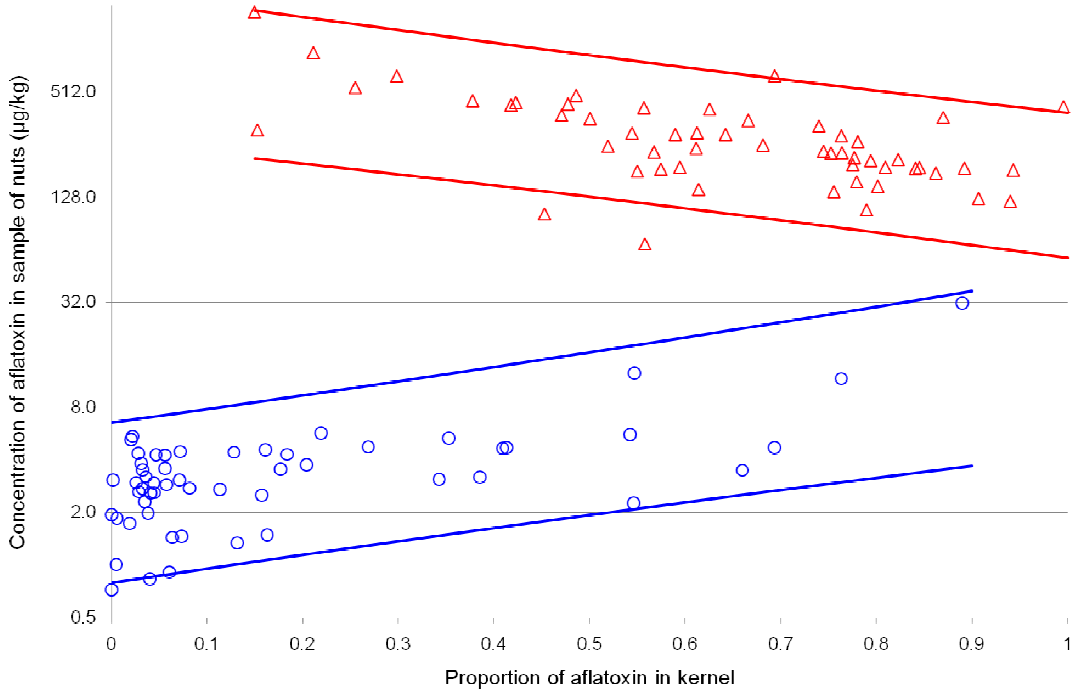


Figure 2: Relation between concentration of aflatoxins in nuts and proportion of aflatoxins in the kernel (good nuts and rotten nuts)



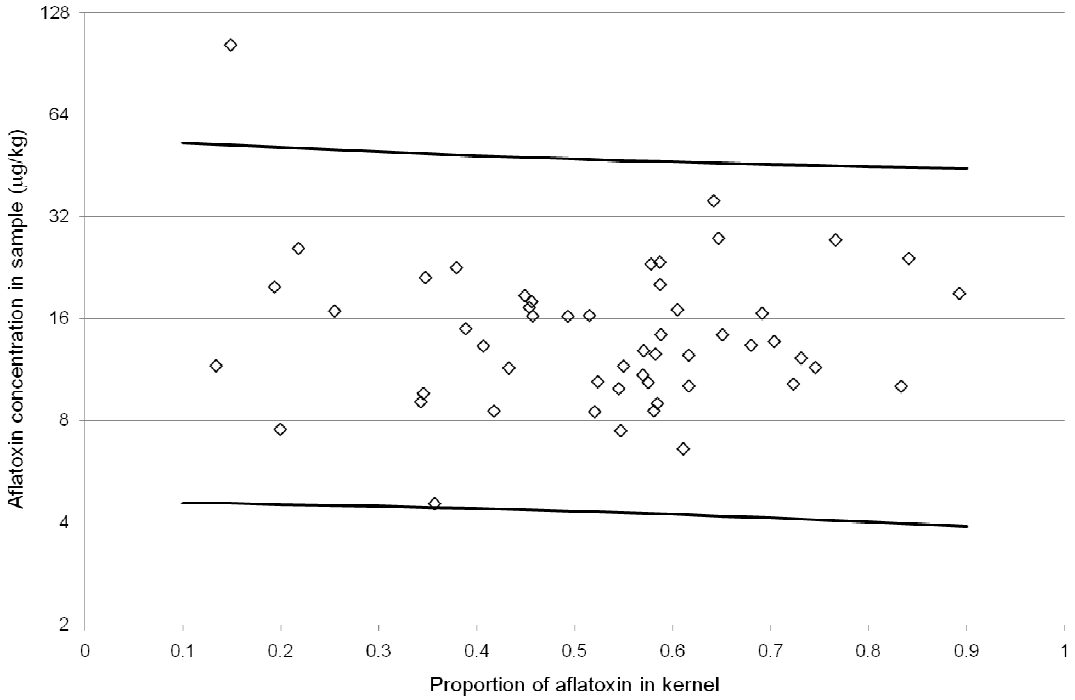
Concentration of aflatoxins in good nuts from samples of at least 30 kg

95% confidence interval for new observations

Concentration of aflatoxins in rotten nuts from sample of at least 30 kg

95% confidence interval for new observations

Figure 3: Relation between concentration of aflatoxins in nuts and proportion of aflatoxins in the kernel (all nuts)



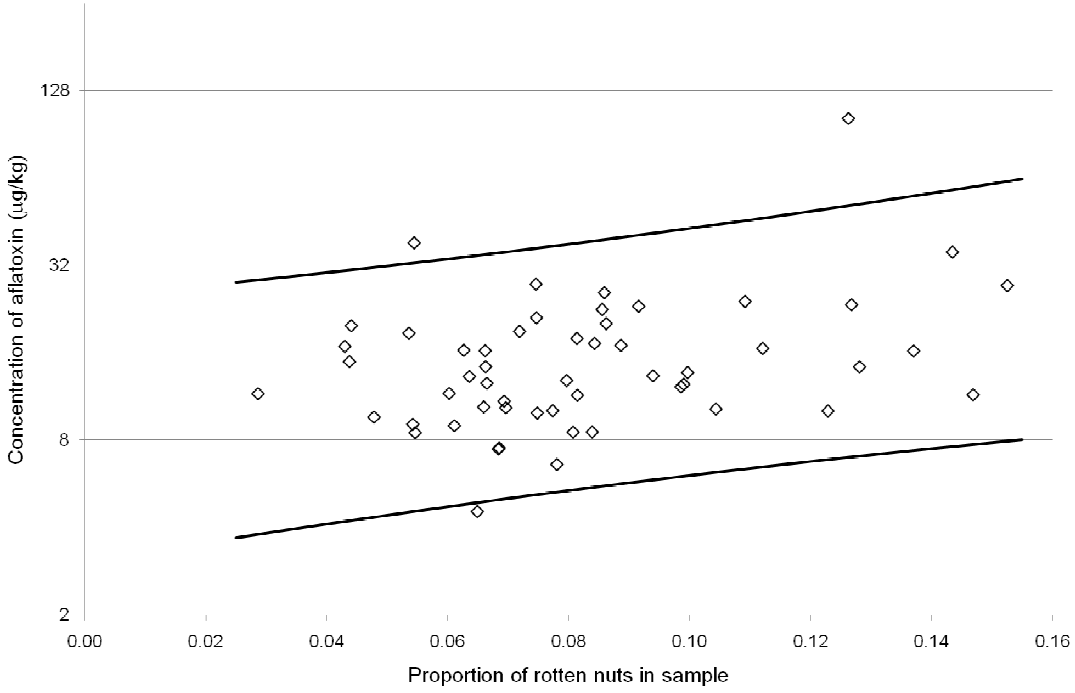
◇ Concentration of aflatoxins in all nuts in samples of at least 30 kg

—— 95% confidence interval for new observations

There was a weak relation between the aflatoxin concentration in the samples and the proportion of rotten kernels in the sample (Figure 4 and Appendix 2). For example we can expect a sample that contains 4% of rotten nuts to contain between 4 and 32 µg/kg of aflatoxins, and we can expect a sample that contains 15% of rotten nuts to contain between 8 and 64 µg/kg of aflatoxins.

Statistical assessment of the data gave an estimated mean conversion factor of 1.05 with a 95% confidence interval from 0.86 to 1.25 (standard deviation 0.098). The value of the conversion factor and its uncertainty derived are shown in Table 1 and Figure 5. Details of the statistical analysis and outputs and the associated uncertainties that led to this factor being derived are given in Appendix 2.

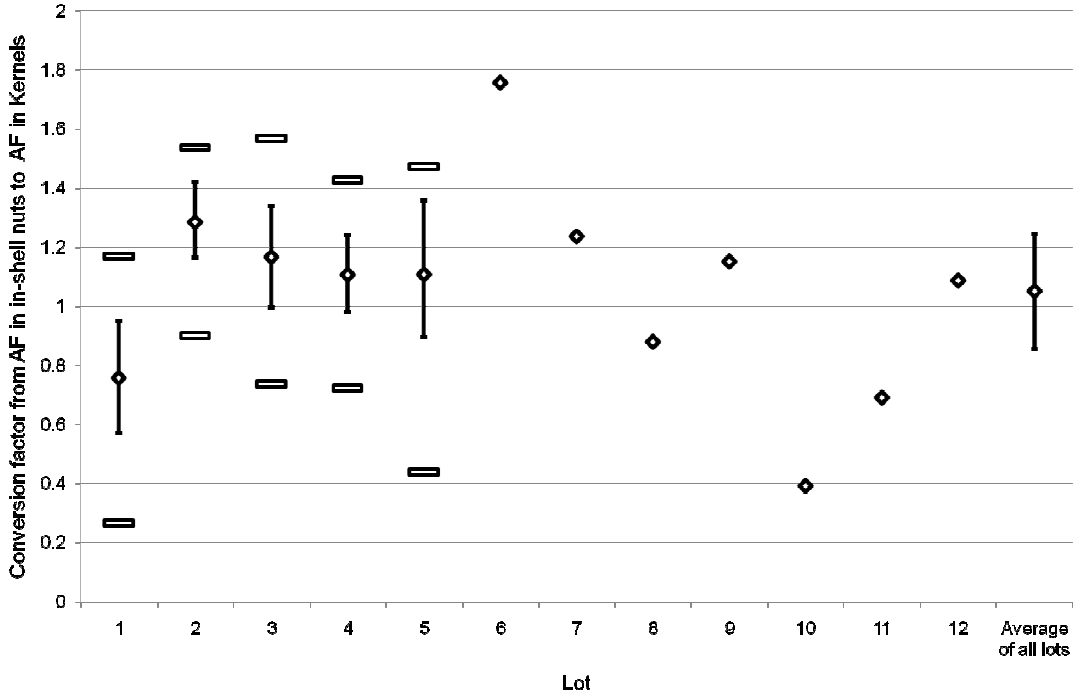
Figure 4: Relation between concentration of aflatoxins and the proportion of rotten nuts



◇ Concentration of aflatoxins in all nuts in samples of at least 30 kg

—— 95% confidence interval for new observations

Figure 5: Observed ranges and bootstrap estimates of the mean conversion factor for lots and mean across lots (all nuts)



◇ Estimated value of factor to convert concentration of aflatoxin in in-shell nuts to concentration of aflatoxin in kernels for lot with 95% confidence interval

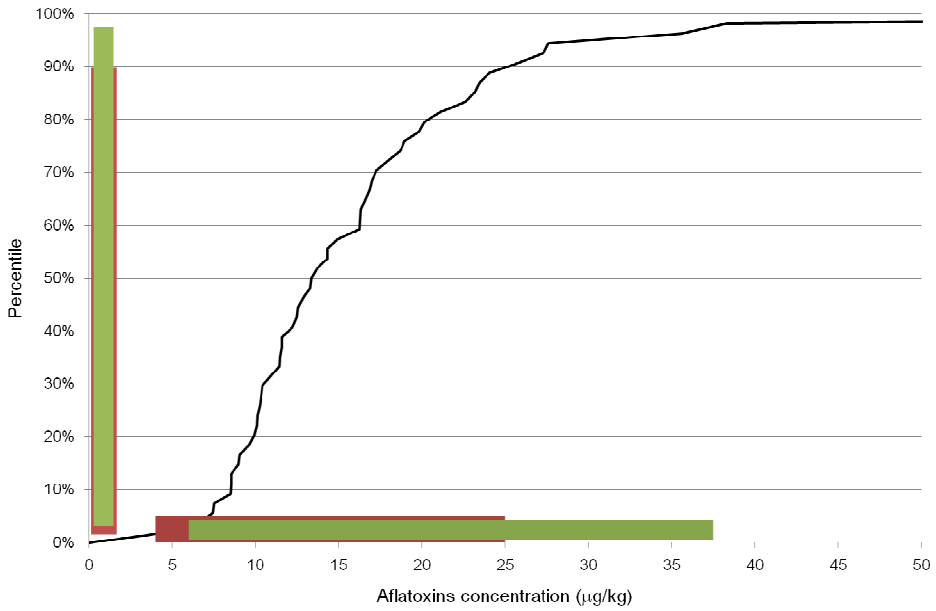
▬ maximum and minimum of conversion factors estimated for each sample in lots

Variation in aflatoxin concentration: sampling uncertainty and limits – The concentration in samples of nuts (good and rotten; shell and kernel) varied with a relative standard deviation of 0.493 between samples within lots and by a relative standard deviation of 0.551 between samples in different lots (calculated using an analysis of variance of log transformed concentrations, Appendix 2). Hence, we can expect the concentration in 30 kg samples to lie somewhere within a factor of 2.5 of the average concentration for a lot (i.e. between C/2.5 and C×2.5 where C is the observed concentration in a sample, based on a log-normal distribution with a relative standard deviation of 0.493)[1]. If the lots examined in this study are representative of all lots then based on the observed

[1] We observed a relative standard deviation of 0.493 for the between-sample, within-lot. Results were right-skewed and approximately log-normally distributed. A RSD of 0.493 on the original scale is equivalent to a standard deviation of 0.467 of the natural-log scale (Appendix 2e). Hence a 95% confidence interval on the natural-log scale is approximately ±0.913 which, when back-transformed to the original scale is equivalent to a factor of 2.5, i.e. taking sampling and analytical uncertainty into account a result may be expected to lie within a factor of 2.5 of the true lot mean.

empirical distribution of CONFORCAST project results an estimated 88% of 30 kg samples will lie within this factor of 2.5 of a limit of 10 µg/kg, and an estimated 94% of 30 kg samples will lie within this factor of 2.5 of a limit of 15 µg/kg (Figure 6) [2].

Figure 6: Cumulative distribution of aflatoxin concentration in samples taken from 12 lots



Estimated distribution of 30 kg samples

Interval within a factor of 2.5 of a limit of 10 µg/kg
(88% of results lie between 4 and 25 µg/kg)

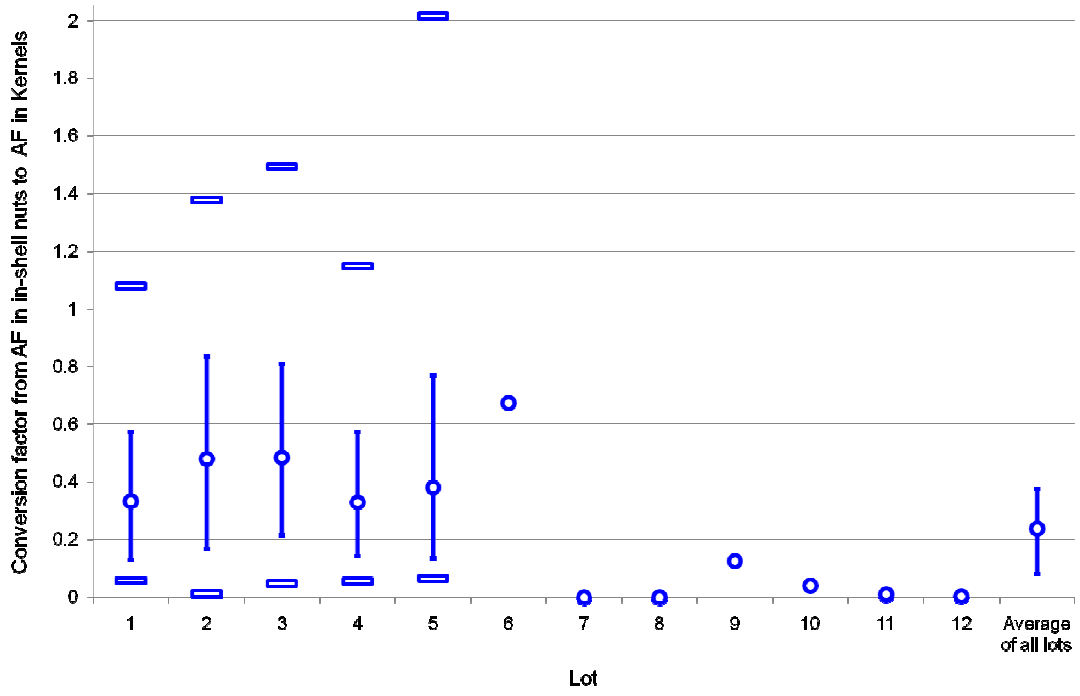
Interval within a factor of 2.5 of a limit of 15 µg/kg
(94% of results lie between 6 and 37.5µg/kg)

i.e. the great majority of samples yield estimates of aflatoxin concentration that are close to legislative limits when sampling variation is considered.

[2] 88% of results lie between 4 and 25 µg/kg. 94% of results lie between 6 and 37.5 µg/kg.

The effect of sorting nuts – The potential effect of sorting nuts was assessed by repeating the examination of the proportion of aflatoxins in the kernel using results produced by the measurement of good nuts only (Table 2, Figure 7). Estimates of the lot conversion factors, for the five lots which had yielded 10 samples, lay between 0.128 and 0.836. The estimated mean conversion factor F for good nuts only was 0.238 with a 95% confidence interval from 0.102 to 0.397. This does not have any practical implications for current control because samples are taken from consignments on an 'un-sorted' basis, i.e. random aliquot samples are taken from throughout the whole consignment. Sorting may take place later in the supply chain when the nuts are subject to further processing.

Figure 7: Observed ranges and bootstrap estimates of the mean conversion factor for lots and mean across lots (good nuts only)



Estimated proportion of aflatoxins in kernels in a lot with 95% confidence interval

Maximum and minimum of conversion factors estimated for each sample in lots

The current practice is to assume that all of the aflatoxins in an in-shell nut is contained in the kernels and to use a conversion factor equal to approximately 2 (based on the observed kernel/shell weight) while also assuming that there is no uncertainty about the value of this factor. For example a result of 40 µg/kg in-shell would be reported as something like 80 ± 35 µg/kg kernels (depending on the observed analytical uncertainty and kernel/shell weight). However, the true kernel concentration in the sample is likely to lie within 42.0 ± 33.6 µg/kg. Hence, while the *reported*

uncertainty may be smaller where a factor of 2 is used, which is assumed to have zero uncertainty, the expected difference between true concentration and estimated concentration is larger compared to that when a factor of 1.05 (that derived here) is used.

Objective 2. Source samples for analysis

The agreed approach was that 2 kg samples would be taken from as many different sources as possible. This was decided for two main reasons. The first was the difficulty encountered when trying to obtain samples of 'known' aflatoxin concentration. The second was the high cost associated with the sample preparation, which in this case would be the hand shelling step. Samples of 10 kg take a large amount of effort to hand shell, which would have increased the cost of the project beyond that of the available budget. The risk of using 2 kg samples was that potentially any model/factor developed in the first part of the project may not be applicable to a 2 kg sample size (this is a risk as 2 kg samples may be expected to produce more variability than 10 kg samples and tends to show more extremes of aflatoxin concentration, i.e. very low and very high aflatoxin concentrations. However all sampling of this nature has a risk associated to it as the aflatoxin concentration cannot be determined until after the sample has been taken. Therefore the 2 kg sample size is a compromise - it is the smallest composite sample size allowed under the EU Regulations (Regulation (EC) No. 401/2006 and Regulation (EU) No. 178/2010), but obviously it is not as large (or as difficult or expensive to handle) as a 10 kg sample. Samples of 'known' aflatoxin levels were preferred to samples purchased 'blind' from retail stores. Buying samples had a high risk of obtaining samples with no detectable aflatoxin, which could not have been used in any statistical validation work. Therefore it was decided and agreed with the FSA that samples with some known history, or that potentially contained aflatoxin should be obtained. Initially the target number of samples was twenty, but this was reduced to eleven when the difficulty of obtaining appropriate samples became apparent.

Extensive efforts were made to obtain samples, and many individuals and organisations were contacted including:

The Association of Port Health Authorities (APHA) and several individual Port Health Authorities were contacted. As a result of this a Fera representative attended the APHA Technical Committee on Food and Feed. A presentation was made and the ports represented asked if they received any in-shell Brazil nuts, and if so could they provide samples. All the ports represented stated they very rarely, if ever, received in-shell Brazil nuts. Some reported they had received them in the past, but after a year with a high number of rejections, they do not receive them anymore. One port reported that they had received samples infrequently and would contact Fera if they received any over the life of the project. Other ports agreed to do the same, however no contact to report any sample arrivals was received. This ties up with evidence from EU import figures that showed no in-shell Brazil nuts were imported into the UK in recent years.

The Nut and Dried Fruit Trade Association (NDFTA) and several of its member companies identified as dealing in in-shell Brazil nuts were contacted. Two companies both directed us to the Chair of the INC Technical Committee, Pino Calcagni, at Besana in Italy.

UK Official Control Laboratories known to have Designated Ports of Entry within their region were also contacted. Two laboratories reported they had rejected a high number of consignments several years ago and had not received any samples since. This supported the information provided by the APHA and information from the Rapid Alert System for Food and Feed (RASFF) reports.

UK retailers were contacted however no samples were provided. Local retail shops were regularly checked for in-shell Brazil nuts. Stores checked included major supermarkets and smaller retailers, including health food stores. Samples were purchased in two supermarkets. None of the other stores was found to stock any in-shell Brazil nuts.

Laboratories in the European Union Reference Laboratory – National Reference Laboratory (EURL-NRL) network for mycotoxins were contacted but only one of the laboratories contacted had received a sample for testing and the sample had been completely used up by this laboratory and so was no longer available.

Of the **contract testing laboratories** contacted one reported that they mainly received samples that were shelled, and in any case all samples they received were prepared into a slurry mixture before analysis (leaving no sample available for analysis). A Brazilian laboratory did not have samples readily available, but did offer to try to obtain samples from local markets. In view of the short time scale and the fact that the history and provenance of the samples would not be known, this offer was declined.

Besana Group in Italy and the INC Scientific Committee agreed to collaborate with the project. Many of the contacts made suggested this group as the most likely source of samples for this project. INC supplied 9 x 2 kg samples, representatively sampled from different contaminated consignments (200 kg each) of in-shell Brazil nuts that had known harvest, storage and transport information. The nuts were of different sizes, and the lots had been tested previously and found to contain aflatoxins in the range of 10 - 100 µg/kg. The lots were retained in case further sampling from them was required at a later stage. Full details, as supplied, of the samples are given in Table 3.

RBiopharm Rhone, Brazil – was contacted. They obtained 2 samples from bulk lots from different locations. Full details, as supplied, of the samples are given in Table 3. Each sample comprised approximately 6 kg of in-shell Brazil nuts.

A total of 11 samples were obtained for the project. A summary of sample information is provided in Table 3.

Objective 3. Assessment of nut-shelling process

The two samples purchased from supermarkets were used for the shelling assessment.

Three types of nut cracker were tested.

1. A traditional squeeze type, with one pinch point that grips and cracks the nuts

2. A traditional squeeze type, with two pinch points
3. 'Top Cracker' – a cup shaped holder that squeezes to crack the nuts

Photographs of all three crackers with sample nuts that were cracked by them are given below (Pictures 1, 2 and 3).

Picture 1.

Picture 2. -

Picture 3.

Information found on the internet suggested different approaches to help shell Brazil nuts cleanly and in one whole piece. Various approaches were recommended, a common suggestion was to soak the nuts in water or heat the nuts in water either on a cooker hob, or in a microwave. This was believed to shrink the nut kernel inside the shell making it easier to crack. The nuts should be cooled and allowed to dry completely before shelling, as any excess moisture could make the nuts more difficult to crack. The use of any protocol involving soaking, steeping, boiling or heating the nuts in water was discounted, due to the potential for the water to wash or extract any aflatoxin from the shell and thus change any potential aflatoxin levels in the shell.

Another suggestion was to roast or bake the nuts in the oven. Various time and temperature combinations were suggested. This approach was also discounted as although from other stability work it could be presumed the aflatoxins themselves would be stable, there is little evidence to support the stability of aflatoxins during such a process. The main concern was how the heat would affect the texture of the nut kernel, and how this would affect the sample grinding, homogenisation and analysis procedures.

Attempts were made to find out how Brazil nuts are shelled commercially. All the information obtained suggested that the nuts are hand shelled by local workers in Brazil nut sorting factories. It seems that a short roasting or baking period is used to make the shells more brittle before the nuts are hand shelled. An experienced worker can shell up to 20 kg of Brazil nuts a day (http://www.thenutfactory.com/kitchen/edible/facts-Brazil.html).

On-line information suggested freezing the nuts, for varying lengths of time, prior to shelling can help by making the shell brittle and separating the kernel from the shell so it can be removed cleanly. This is suggested for domestic hand shelling. The cold temperature apparently helps to separate the kernel from the shell, and makes the shell more brittle and easier to crack. This approach seemed to have the most merit and so this was assessed.

Three different conditions were assessed:

1. Nuts stored at ambient temperature
2. Nuts stored overnight in a freezer (temperature range -18 to -20°C)
3. Nuts stored overnight in a -80°C freezer

All temperature conditions were assessed with all three nut crackers.

Room temperature – Nut cracker 1

The first nut cracked was brown and smelly, and had obviously deteriorated badly. It also did not crack cleanly, with shell and kernel fragments mixed together (Picture 4). This was the most difficult and time consuming way to shell nuts. Subsequent nuts that were shelled with this cracker generally broke or were compressed or damaged to some degree. Picture 5 shows a nut that didn't break in too many small pieces, however a significant amount of kernel was left attached to the shell. If this happened regularly during the shelling of the project samples, it would take a lot of time to separate the components, and there would be a potential risk of cross contamination between shell and kernel that might add some uncertainty to the results. Picture 6 shows how badly some nuts fragmented using these conditions.

Picture 4.

Picture 5. -

Picture 6.

Room temperature – Nut cracker 2

This cracker gave much better results at room temperature. Although some kernels broke, they did separate cleanly from the shell (Picture 7).

Picture 7.

Room temperature – Nut cracker 3

This type of cracker uses a slightly different mechanism to crack the nuts (Picture 8). The nut sits in a cup and when the handles are squeezed the ridges inside the cup exert pressure and crack the shell. Using this cracker, even at room temperature, the nuts cracked easily and were cleanly separated from the shell resulting in whole kernels (Picture 9).

Picture 8.

Picture 9. -

Nuts stored at -20°C, crackers 1, 2, and 3

Freezing the nuts led to mixed results. For cracker 1 in some cases the nuts shattered and did not separate cleanly from the shells, in some cases the nuts separated cleanly and gave whole kernels (Picture 10). For cracker 2, the kernels did tend to break, but were mostly separated cleanly from the shell (Picture 11). For cracker 3 the results were a bit more variable than at room temperature, the majority of the nuts separated cleanly and in one whole piece, however a few did disintegrate to small pieces and fragments were stuck to the shell (Picture 12).

Picture 10.

Picture 11. -

Picture 12.

Nuts stored at -80°C, crackers 1, 2, and 3

For cracker 1, the results were variable. In the majority of cases the nuts shelled cleanly and in one piece, but on occasion some nuts broke up (Picture 13). For cracker 2, the results were better with the nuts breaking consistently and cleanly into whole nuts (Picture 14). For cracker 3, the results were consistently good, with whole nuts cleanly separated from the shell in all cases (Picture 15).

However there are potential problems with dealing with nuts that have been stored at such a low temperature. Firstly they were very cold to handle and the use of protective gloves required for the personnel to prevent 'freezer burn' may mean they cannot carry out the task easily. The samples quickly acquire a lot of ice condensation; this can be seen in the pictures. This addition of water may make it more difficult to grind the samples, in particularly the shells. They also do not retain the

temperature so would need to stored at the -80°C until immediately before shelling, meaning only - small amounts could be shelled at a time – again adding to the time required for the whole process. -

Picture 13.

Picture 14. -

Picture 15.

The results showed consistently good performance for cracker 3 at all temperatures, although the results were not as good at -20°C as room temperature or -80°C. Nut cracker 1 did not perform well at any temperature and was awkward to use, nut cracker 2 gave good performance at the cold temperatures. Taking into consideration the potential problems from handling nuts at extreme low temperatures, and the fact that results from normal freezer temperatures were slightly worse than those found for room temperature it was decided to proceed with shelling of the project samples with cracker 3 for nuts at room temperature. This also had the merit that the simpler procedure avoided potential effects that heating, freezing, soaking etc. may have had on the distribution of aflatoxins between the shell and the kernel surfaces. Shelling by hand at room temperature and using no special pre-treatment of Brazil nuts is by far the most common practice by UK consumers.

Objective 4. Hand shelling and sample preparation

Although the initial assessment using UK shop bought Brazil nuts showed the nuts separated cleanly from the shells, in practice this was not always the case. Efforts were made to clean or scrape any residual nut pieces from the shells, however this was not always possible. In many cases the residue was an oily liquid that coated the inside of the shell or fragments of skin and kernel tightly bound to the shell and it was not possible to completely remove it. Therefore it was decided that samples

would be sorted into three groups; shells with no visible nut residue, shells with residue and all kernels. All eleven samples were treated in this way. The weights of all samples were recorded and are give in Table 4. For consistency, the 2 samples received directly from Brazil were randomly sub-sampled, to produce a 2 kg subsample for each, so all 11 samples used in the study were the same size.

It seemed to be the case that if a nut was obviously spoiled or bad then it was much more difficult to separate the kernel from the shell. In particular as the samples were shelled it could be seen that the brown 'skin' surrounding the kernel was often fused onto the inside surface of the shell. This was impossible to separate from the shell. The shape, form and quality of individual nuts ranged from whole perfect looking kernels, through various stages of discolouration, small size (could be heard rattling in the shell) as small as a pea, through to the kernel being liquid or the shell being completely empty. It must be presumed these sample lots contain higher levels of bad nuts as they had already been found to be contaminated whereas the shop bought nuts were better quality. In Table 4, proportion of shell and kernel from each sample has been calculated. The shell ranged from 50.9% to 57.9% of the total nut weight. For several of the samples from Besana the percentage of shell is higher than the average of 53% reported in DeMello and Scussel (2007), or 49.8% reported by Vargas *et al*. (2011). It was noted above that the percentage of shell (and so the F-factor) increases as healthy nuts spoil and the kernel weight decreases. The samples Bulk 1 and Bulk 2 fell within these published ranges, these were the freshest samples having been harvested most recently. As all kernels were collected together an accurate assessment of the number of 'good' nuts and 'bad' nuts was not made. However the nuts had such a powerful rancid smell they had to be handled in a fume cupboard. The estimated percentage of bad nuts in the 9 Besana samples was at least 50%. For the two Bulk samples direct from Brazil the number of bad nuts was much lower (no more than 10-20%).

Samples had been stored at ambient temperature prior to shelling (as per information provided by sample suppliers), but were immediately stored in a freezer after shelling.

Objective 5. Sample analysis of independently sourced Brazil nut samples for aflatoxins

Sample analysis was carried out at Fera in the order of clean shells, shells with residue, then kernels. This was done to prevent cross contamination or carry over in case any of the kernel samples were very highly contaminated, as they had been observed to be in a poor state during shelling and preparation.

In all cases calibration curves and other system suitability parameters met UKAS criteria. Values found for in-house reference materials were within the acceptable range for the material. Recovery data was lower than normal specified limits for aflatoxin B_1 and G_1 for the kernel analysis, with average values of 54, 72, 55 and 78% obtained for aflatoxin B_1, B_2, G_1 and G_2 respectively, for samples spiked at 10 µg/kg each aflatoxin. This can be partly explained by the fact that no known blank material was available for spiking and spikes were made into the reference sample that already contained aflatoxin. A higher spiking concentration could have led to higher recovery in these conditions, although the discrepancy between the recovery for B_1 and G_1 and B_2 and G_2 may

indicate there was some problem with the derivatisation for these analytes in the samples that was not mirrored in the calibration samples.

The results for the shells also show recovery less than 70%, with average (n = 6 to 8) values of 58, 51, 60 and 56% respectively for aflatoxins B_1, B_2, G_1 and G_2 at a spike level of 10 µg/kg. Again no known blank was available and therefore a clean shell sample from the one of the bulk samples from Brazil was selected for spiking. This was done as there was no other test sample available in the laboratory that was similar to the Brazil shells. The duplicate values for all but one sample, including those found to contain high levels show very good agreement. The sample that did not have good agreement (3966 kernels) showed good agreement for the shell portions.

All data was initially reported not corrected for recovery. This was for a number of reasons. The data from the 'CONFORCAST' project (used to develop the model in Objective 1) was not corrected for recovery so it was reported in the same format as that. Also the spiking level was 10 µg/kg, but the levels found in many cases were significantly higher than that. Under normal circumstances (and following the UKAS accredited procedure) a correction factor would not be applied to samples at such high concentrations, as the method performance at 10 µg/kg may not be the same as it is at concentrations over 1 mg/kg. However recovery information was supplied for the 'CONFORCAST' data, and all recovery and QC information were supplied with the Fera data for the statistical evaluation. It was considered important to apply the correction factors before the data was used in any statistical or modelling work as Regulation limits are based on corrected values and the use of uncorrected data could influence the final statistical output. A summary of the data obtained for the independently sourced samples analysed at Fera is given in Table 5.

It was observed that although the samples received at Fera had appeared spoiled, with a high number of 'bad' nuts and would not have been consumed, they did not all contain high levels of aflatoxins. This demonstrates that the spoilage of nuts is not entirely caused by aflatoxigenic moulds, but can be caused by a wide range of spoilage moulds and bacteria (Freire *et al*., 2000 and Johnsson *et al.*, 2008). In fact four of the eleven kernel samples did not contain aflatoxins above the limit of quantification (0.2 µg/kg each aflatoxin). The shells of these samples contained very low levels of aflatoxin. In all cases where the aflatoxin level in the kernels was high, although aflatoxin was detected in the shell it would have made a minor contribution to the overall aflatoxin concentration in a whole 'in-shell' sample. For example sample 3929 contained over 5 mg/kg aflatoxin in the kernels, but only approximately 0.1 mg/kg in the shell. The same pattern was observed for all the highly contaminated samples, i.e. the kernels contained very significantly higher concentrations of aflatoxin than the corresponding shells. It is also interesting to note that in most cases in the CONFORCAST data a similar pattern was observed (Appendix 1), at low levels while the shell contained more aflatoxin than the kernel it was on the whole not of sufficiently high concentration to make the sample non-compliant, while at high levels on average the kernels contained more aflatoxin than the shells.

The raw data, as well as the recovery data and the results of the in-house reference material samples were used for statistical analysis and comparison with other data supplied earlier in the project (Objective 6).

Objective 6. Validate / test model using data from Objective 5

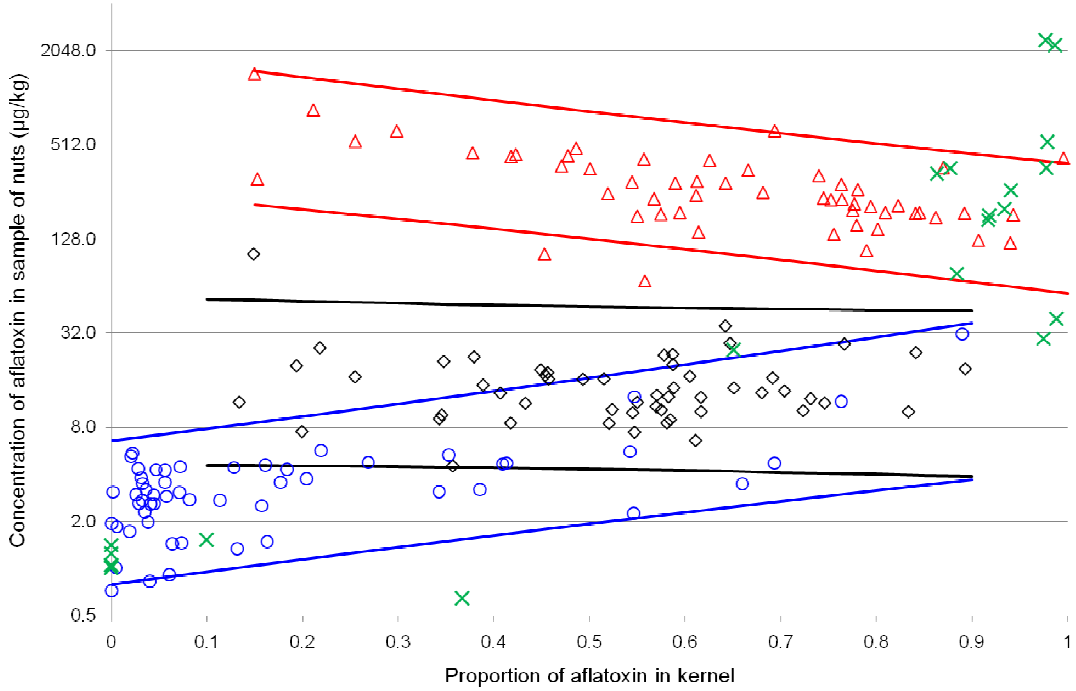
The relation between the concentration of aflatoxin in in-shell (whole) Brazil nuts and the concentration in the kernels was assessed in Objective 1 and a conversion factor of 1.05 was proposed. Results obtained by the analysis of independently sourced Brazil nuts (Table 3) did not consistently lie in the ranges estimated using the CONFORCAST data (Appendix 1), shown in Figure 8. In both the Fera and the CONFORCAST studies the majority of the results fell into two clusters: a low concentration, low kernel aflatoxin content cluster; and a high concentration, high kernel aflatoxin content cluster. However, results produced during the two studies are not directly comparable for two reasons:

Firstly there is the observation that these 2 kg samples appeared to contain a particularly high proportion of rotten nuts: they were not representative of generally edible nuts. The mean aflatoxin concentration of the 2 kg samples was a little over 300 µg/kg with 96% of aflatoxins in the kernel. Hence, while the mean is consistent with the profile from the CONFORCAST results for rotten nuts, this is effectively a single observation of about 40 kg of nuts, so very weak evidence (for a single observation at that concentration a range from 15% to 100% is consistent with CONFORCAST data).

Secondly there was a difference in sample mass (at least 30 kg for the CONFORCAST study but only 2 kg for the independently sourced nuts). Small samples, where each sample comes from a different lot, are difficult to compare with results produced by larger samples from different lots because there is a small number of observations from skewed distributions (for factor and very skewed for concentration) which tend to give biased (downwards) estimates of mean and variance for these quantities.

Therefore it was not possible to validate or refute the conversion factor (model) derived in Objective 1.

Figure 8: Comparison between CONFORCAST data and analytical results from independently sourced Brazil nuts



Concentration of aflatoxins in good nuts from samples of at least 30 kg

95% confidence interval for new observations

Concentration of aflatoxins in rotten nuts from sample of at least 30 kg

95% confidence interval for new observations

Concentration of aflatoxins in all nuts in samples of at least 30 kg

95% confidence interval for new observations

Concentration of aflatoxins in all nuts from 2 kg independently sourced samples

5. CONCLUSIONS

The most comprehensive data supplied for use in this project was that of the CONFORCAST project. Assessment of this data established that for 'good kernels', in the majority of cases, the aflatoxin level was lower than that of the corresponding 'good shells', while 'rotten kernels' (on the most part) contained significantly higher concentrations of aflatoxin than the corresponding 'rotten shells'.

Of the eleven independently sourced samples analysed at Fera, four of the kernel samples did not contain aflatoxins above the limit of quantification (0.2 µg/kg each aflatoxin) despite the samples having a 'bad' appearance. The shells associated with these samples contained very low levels of aflatoxin, and none would have exceeded the current (or the previous) limit for aflatoxin. In all cases where the aflatoxin level in the kernels was high, although aflatoxin was detected in the shell, it would have made a minor contribution to the overall aflatoxin concentration in a whole 'in-shell' sample. For example sample 3929 contained over 5 mg/kg aflatoxin in the kernels, but only approximately 0.1 mg/kg in the shell. The same pattern was observed for all the highly contaminated samples, i.e. a similar pattern to that generally observed for CONFORCAST data. Therefore data from both studies supports the conclusion that initially the contamination occurs on the shell, or more particularly on the surface of the kernel at the interface with the shell, with little contamination on the kernel. However as the infection increases the amount of aflatoxin on the kernel increases dramatically and the level of aflatoxin on the shell remains at a relatively low level. This is supported from work carried out as part of the SAFENUT project (STD Project 114 Final Report, 2008). This makes sense biologically as other reports have highlighted the critical time for contamination to occur as being the period between when the nuts are harvested and when they are stored and dried prior to introduction to sorting factories. Typically drying takes place in the forest. The SAFENUT project (STD Project 114 Final Report, 2008) highlighted the drying stage as a critical control point to prevent contamination. If drying is not carried out properly nuts either retain too high a water activity allowing mould growth, or if dried too quickly in the sun the shells can split and crack allowing infection to occur. It makes sense that initial mould growth on the shell is slow and therefore aflatoxin contamination limited, as the shell is a fairly poor substrate (nutrient source) for mould growth. However as the infection develops and the mould mycelia grow into the kernel it is not unexpected that the mould will grow rapidly and produce large amounts of aflatoxin as the kernel is a rich source of nutrients. In particular, if this occurs in the ambient temperatures and humidity of the rain forest which are well documented as the ideal conditions for aflatoxin production. Some of the aflatoxin produced on the kernels may also diffuse back into the shell as the kernel deteriorates; it was observed some spoiled samples were reduced to a liquid state inside the shell. Vargas *et al*. (2011) have suggested that analysis of Brazil nuts should be carried out after nuts have been sorted to remove rotten nuts.

Considering the underlying biological processes, therefore, in principle, it should be possible to derive the relative proportions of aflatoxin distribution between the kernel and the shell, at the crucial decision point when the kernel is at the legal limit of 10 µg/kg, and thereby establish a tailored correction factor to be used if in-shell samples are tested. However given the aforementioned severe inhomogeneity of contamination, this is too simplistic. A representative sample of nuts (e.g. 10 kg) could contain just one highly contaminated nut (with relatively little aflatoxin associated with the shell) or could contain several moderately-contaminated nuts (with

proportionately more of the aflatoxin associated with the shell) but with the two cases returning the same analytical result as the average for the 10 kg of nuts. The correct correction factor would be different for the two cases even though both would be at the decision point of legally compliant or not.

Statistical evaluation of the CONFORCAST data derived a mean factor of 1.05 to describe the relation between the observed concentration of aflatoxins in whole nuts and the concentration of aflatoxins in kernels. While there was variation in the value of the factor between and within lots, there was no consistent trend with concentration for samples of at least 30 kg which contained between approximately 4 and 100 µg/kg of aflatoxins, taken from lots which contained between approximately 10 and 30 µg/kg of aflatoxins. However as the relative levels in the shell and kernel change over time (i.e. as infection increases) then it is unlikely that it will be possible to define a single conversion factor that will always predict the concentration of aflatoxin in the kernel without some degree of uncertainty. Indeed the removal of rotten nuts produced samples with a reduced mean proportion of aflatoxin in the kernel and a lower value for estimated conversion factor (95% confidence interval between 0.102 and 0.397).

If the results provided by the CONFORCAST project are representative, then we can expect many lots to contain aflatoxins that are close enough to legislative limits so that sampling uncertainty may lead to measurement results that could be either side of the limit. In addition the use of measurement of in-shell nuts to estimate the concentration of aflatoxin in kernels for control purposes increases the reported analytical uncertainty from approximately ± 44% (as would be predicted from Horwitz for direct measurement of kernels) to ± 80%, but reduces the expected difference between true concentrations and reported concentrations compared to the use of a factor '2' because estimates are less biased.

The analytical measurement and the conversion factor each have an associated uncertainty. Where a measurement is used to estimate the concentration of aflatoxins in the kernels of nuts in a particular lot the analytical uncertainty is typically estimated to be ± 44%. The results examined in this study suggest that the uncertainty associated with the estimated concentration of aflatoxins in the kernel of a lot based on an in-shell measurement result converted to a kernel concentration using a factor of 1 is ± 80%. The main source of additional uncertainty being between-sample variation in the proportion of aflatoxins in the kernel and on the shell.

Current practice for assessment against the legislative limit is to measure aflatoxins in in-shell samples, then correct this for analytical recovery. The analytical measurement uncertainty associated with the result is estimated, , e.g. 10 ± 4.4µg/kg. It is assumed that all measured aflatoxins are in the kernel and a conversion factor of approximately 2 (with no associated uncertainty) is applied, which in effect doubles the concentration and its associated uncertainty, e.g. 10 ± 4.4µg/kg becomes 20 ± 8.8 µg/kg. Then if the converted result is unequivocally above the limit when the converted analytical uncertainty is taken into account the sample is declared to be non-compliant. The results of this study show that this is likely to lead to estimates of concentration in the kernel which are biased upwards and with an estimated uncertainty that is too small. For example, this study shows that an in-shell result of 9 µg/kg is consistent with a kernel concentration of 1.8 to 16.2 µg/kg (conversion factor of 1 and combined uncertainty of ± 80%), i.e. non-compliance is not demonstrated. However under current practice the kernel concentration would be reported

as 18 ± 7.9 µg/kg (conversion factor of 2 and analytical uncertainty of ± 44%), a result that demonstrates non-compliance.

Where in-shell measurements are used to estimate the concentration of aflatoxins in kernels, the results examined in this study show that variation in proportion of aflatoxins in the kernel and shell means that a result of approximately 50 µg/kg in-shell is needed in order to demonstrate that kernels are non-compliant at a level of 10 µg/kg.

A third option is to measure the concentration of aflatoxins in shelled kernels. This reduces the expected uncertainty about the concentration of aflatoxins in kernels from ± 80% to ± 44% at the cost of increased sample preparation. Here a result of 18 µg/kg can be expected to demonstrate non-compliance.

Measurement of aflatoxins in independently sourced Brazil nuts gave results outside the range of values produced by samples of nuts that were used to estimate the value of the conversion factor. Their mean was consistent with the profile of rotten nuts produced by CONFORCAST data. However, because the independently sourced Brazil nuts were not considered to be generally edible, and because they were much smaller samples, single 2 kg samples and the 'CONFORCAST' samples were at least 30 kg, it was not reasonable to use these results to validate or refute the value of the estimated conversion factor.

The expected mean value of the conversion factor for smaller samples is the same as that for larger samples. However the within-lot variation in the observed value of the factor for small samples will be larger, and crucially is likely to be more skewed, than the variation for larger samples. Hence, the uncertainty associated with estimates for the kernel concentration for samples from single lots using a conversion factor applied to smaller samples will be larger than the ± 80% observed in this study. Hence, if larger uncertainties (> ± 80%) are not acceptable, where the smallest samples are used for control (e.g. the 2 – 12 kg sample size currently specified in Commission Regulation (EU) No. 178/2010), shelling, or the use of a conservative factor (e.g. 2) may be the most reasonable option.

However, if the use of a conversion factor for smaller samples may be useful then a study based on sampling and measuring the concentration of aflatoxins in shells and kernels similar to that undertaken in the CONFORCAST project is one way that the relation between shell and kernel concentration in these smaller samples could be studied. For example, changes to legislation in 2010 now require that a maximum sample of 20 kg is taken for enforcement or control. This is required to be split into 2 x 10 kg subsamples before homogenisation and analysis. Therefore it would be preferable to undertake work similar to CONFORCAST, using 10 kg samples, ideally taken at import in a control situation and for as many different consignments as possible.

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Table 1: Estimates of the value of the factor for converting between the concentration of aflatoxins in in-shell nuts and the concentration of aflatoxins in the kernels (all nuts)

Lot	Estimate	95% confidence interval for estimate		Maximum and minimum observed factor	
1	0.758	0.566	0.946	0.267	1.169
2	1.285	1.150	1.406	0.902	1.538
3	1.167	0.995	1.339	0.736	1.569
4	1.107	0.974	1.234	0.723	1.426
5	1.108	0.859	1.322	0.439	1.473
6	1.757	NE	NE	NA	NA
7	1.237	NE	NE	NA	NA
8	0.879	NE	NE	NA	NA
9	1.152	NE	NE	NA	NA
10	0.392	NE	NE	NA	NA
11	0.691	NE	NE	NA	NA
12	1.088	NE	NE	NA	NA
Average of all lots	1.052	0.859	1.246		

NE: Not estimated, NA, Not applicable

Table 2: Estimates of the average proportion of aflatoxins in the kernels of nuts (good nuts)

Lot	Estimate	95% confidence interval for estimate		Maximum and minimum observed factor	
1	0.332	0.128	0.572	0.057	1.079
2	0.479	0.166	0.836	0.011	1.376
3	0.484	0.212	0.806	0.048	1.494
4	0.329	0.141	0.572	0.054	1.148
5	0.380	0.134	0.767	0.064	2.015
6	0.674	NE	NE	NA	NA
7	0.000	NE	NE	NA	NA
8	0.000	NE	NE	NA	NA
9	0.125	NE	NE	NA	NA
10	0.040	NE	NE	NA	NA
11	0.010	NE	NE	NA	NA
12	0.003	NE	NE	NA	NA
Average of all lots	0.238	0.102	0.397		

NE: Not estimated, NA, Not applicable

Table 3: Sample details

Sample #	Size* (count)	Origin	Date of harvest	Place of drying	Storage at origin	Date of shipment	Date of arrival	Storage in Europe
3552	40/55	Bolivia	01/10	Factory	Ambient	08/06/2010	03/07/2010	Ambient
3424	55/65	Bolivia	01/10	Factory	Ambient	06/05/2010	21/06/2010	Ambient
3929	___	Amazonia – Brazil	02/10	Forest	Ambient	11/06/2010	10/07/2010	Ambient
3420	40/55	Acre – Brazil	02/10	Factory	Ambient	27/05/2010	21/06/2010	Ambient
3939	___	Rondonia – Brazil	02/10	Forest	Ambient	06/05/2010	21/06/2010	Cold
3966	___	Rondonia – Brazil	02/10	Forest	Ambient	11/06/2010	12/07/2010	Cold
3422	40/55	Acre – Brazil	02/10	Factory	Ambient	06/05/2010	21/06/2010	Ambient
3932	55/65	Parà – Brazil	02/10	Factory	Ambient	18/06/2010	19/07/2010	Ambient
3931	40/55	Parà – Brazil	03/10	Factory	Ambient	30/05/2010	12/07/2010	Ambient
Bulk 1	___	Ariquemes, Rondônia Brazil	02/11	Forest	Ambient	17/03/2011	22/03/2011	Ambient
Bulk 2	___	Brasiléia, Acre, Brazil	01-02/11	Forest	Ambient	17/03/2011	22/03/2011	Ambient

* The size/count was not given for all samples. This is a grading system and is based on the number of nuts per pound weight. One pound (lb) = 454 grams. The accepted range of counts for in-shell Brazil nuts per pound is 35/40 (extra large), 40/45 (large), 50/55 (extra medium), 57/62 (medium) and over 70 (small). Some of the counts given do not match these criteria, but the size of the nuts would be classified as medium or extra medium.

Table 4: Weights of samples and shell and kernel portions

Sample #	Sample weight (g)	Weight Kernels (g)	Clean shells (g)	Shells with residue (g)	Total Shell (g)	Total Mass (g)	% Shell	% Kernel
3552	2053	929	423	656	1079	2008	53.7	46.3
3424	2064	837	158	993	1151	1988	57.9	42.1
3929	2039	851	272	845	1117	1968	56.8	43.2
3420	2058	933	187	874	1061	1994	53.2	46.8
3939	2068	878	266	832	1098	1976	55.6	44.4
3966	2041	899	257	860	1117	2016	55.4	44.6
3422	2037	884	125	986	1111	1995	55.7	44.3
3932	2088	877	212	976	1188	2065	57.5	42.5
3931	2042	913	216	892	1108	2021	54.8	45.2
Bulk 1	2040	1098	717	421	1138	2236	50.9	49.1
Bulk 2	2049	945	446	585	1031	1976	52.2	47.8

Table 5: Results of Fera aflatoxin measurements in independently sourced samples (recovery corrected), provided by Besana group and INC (9 x 2 kg samples) and R-Biopharm Rhone, Brazil (2 x 2 kg sub-sampled samples)

Sample #	Aflatoxins in Kernels (µg/kg)					Mass (g)	Aflatoxins in Clean Shells (µg/kg)					Mass (g)	Aflatoxins in Shells with Residue (µg/kg)					Mass (g)
	AFB1	AFB2	AFG1	AFG2	Total		AFB1	AFB2	AFG1	AFG2	Total		AFB1	AFB2	AFG1	AFG2	Total	
3420	469	18	610	27.5	1124	933	1.2	<0.2	0.9	<0.2	2.1	187	11	1.1	13	1.1	26	874
3420	307	15	421	22.1	765	933	1.2	<0.2	0.5	<0.2	1.8	187	7.8	0.9	8.9	0.9	19	874
3422	<0.2	<0.2	<0.2	<0.2	<0.8	884	2.1	<0.2	1.1	<0.2	3.2	125	0.5	<0.2	<0.2	<0.2	0.5	986
3422	<0.2	<0.2	0.5	<0.2	0.5	884	1.6	<0.2	0.7	<0.2	2.3	125	0.5	<0.2	<0.2	<0.2	0.5	986
3424	<0.2	<0.2	<0.2	<0.2	<0.8	837	1.6	<0.2	0.7	<0.2	2.3	158	1.4	<0.2	0.7	<0.2	2.1	993
3424	<0.2	<0.2	<0.2	<0.2	<0.8	837	1.2	<0.2	0.4	<0.2	1.6	158	<0.2	<0.2	<0.2	<0.2	0.0	993
3552	<0.2	<0.2	<0.2	<0.2	<0.8	929	1.6	<0.2	1.2	<0.2	2.9	423	0.7	<0.2	0.5	<0.2	1.2	656
3552	<0.2	<0.2	<0.2	<0.2	<0.8	929	2.0	<0.2	1.4	<0.2	3.4	423	1.1	<0.2	1.1	<0.2	2.1	656
3929	2730	2.9	2680	1.2	5416	851	2.3	<0.2	2.3	<0.2	4.6	271	44	6.1	69	9.6	128	844
3929	2679	3.7	2415	1.8	5100	851	3.0	<0.2	2.9	<0.2	5.9	271	27	2.9	34	3.0	69	844
3931	177	8.6	217.8	9.4	413	913	2.5	<0.2	1.6	<0.2	4.1	216	9.8	0.7	17	1.2	29	892
3931	140	8.0	188.9	5.5	343	913	2.7	<0.2	1.6	<0.2	4.3	216	11	0.7	18	1.1	31	892
3932	<0.2	<0.2	0.4	<0.2	0.4	877	0.5	<0.2	0.5	<0.2	1.1	212	1.6	<0.2	1.1	<0.2	2.7	976
3932	<0.2	<0.2	<0.2	<0.2	<0.8	877	0.5	<0.2	0.7	<0.2	1.2	212	1.1	<0.2	0.9	<0.2	2.0	976
3939	251	36	325.1	37.1	650	878	2.1	<0.2	1.2	<0.2	3.4	266	47	7.1	47	6.4	108	832
3939	301	34	349.4	33.7	718	878	1.8	<0.2	1.2	<0.2	3.0	266	47	6.2	46	5.7	105	832
3966	34	2.3	<0.2	<0.2	37	899	2.1	<0.2	0.9	<0.2	3.0	257	17	1.2	1.2	<0.2	20	860
3966	142	9.8	<0.2	<0.2	152	899	1.8	<0.2	0.9	<0.2	2.7	257	18	1.4	1.2	<0.2	20	860
Bulk 1	158	7.0	329.1	7.5	502	1098	5.9	<0.2	8.7	<0.2	14.6	717	25	0.7	31	1.1	58	421
Bulk 1	128	3.9	198.4	7.1	338	1098	3.0	<0.2	4.1	<0.2	7.1	717	26	0.9	38	1.6	67	421
Bulk 2	33	2.3	38.0	1.2	75	945	<0.2	<0.2	<0.2	<0.2	0.0	266	0.7	<0.2	0.7	<0.2	1.4	585
Bulk 2	34	5.0	15.5	<0.2	55	945	<0.2	<0.2	<0.2	<0.2	0.0	266	1.2	<0.2	1.1	<0.2	2.3	585

APPENDIX 1

An overview of the CONFORCAST project and measurement results

Thirteen lots of in-shell Brazil nuts (4-8 tons) from the states of Para and Acre, classified as ready for marketing and suspected of aflatoxin contamination, were identified for the CONFORCAST study. Nine of the lots were classified as medium and 4 as large category nuts. An aggregate sample (400 kg) was randomly taken from each lot. Each aggregate was mixed and divided into ten in-shell test samples of approximately 40 kg each (130 samples in total). Each sample was hand shelled and sorted by trained women working in the Brazil nut processing plants. Samples were separated into five fractions: good kernels, rotten kernels, good shells with kernel residue, good shells without kernel residue, and rotten shells. For the CONFORCAST project the following definitions were used (as given in Vargas *et al*., 2011):

Fractions: Any part of Brazil nut derived from the shelling and sorting

Rotten kernels: nuts easily segregated visually by a consumer (empty, mouldy, fermented, cut, rotten or black)

Rotten shells: shells from rotten kernels

Good kernels: kernels with no visible damage not rejected by consumers

Good shells with kernel residue: shells from good kernels that had part of the kernel attached

Good shells without kernel residue: shells from good kernels that had no residue attached

Rotten nuts: mass balance of rotten kernels and rotten shells

Good nuts: mass balance of good kernels and good shells

For the study 10 samples from each of 5 lots, and one sample from each of the remaining eight lots were identified for aflatoxin analysis, resulting in 58 test samples. Four samples (lot 13, and one each from lots 2, 3 and 4) were missing due to some fractions not being collected at source. As full mass balance calculations could not be carried out for these samples they were omitted from the data set. Therefore full results were received for 54 samples (216 data points). All data provided is here, and is the same as that presented in Vargas *et al*. (2011).

Despite using a five fraction system in the project description, the data that was supplied and published was classified into four parts as good shells (with and without residue) were combined into one group, thus giving the groups below. The total aflatoxin concentration was measured in each of the four groups taken from each sample:

- Good kernels
- Good shells

- Rotten kernels
- Rotten shells

The mass of each group was also recorded which enabled the concentration and the absolute amount of aflatoxin in the combined sample and parts thereof to be reconstructed.

Appendix 1a: CONFORCAST sample weights

		Sample mass (kg)						
Lot	Sample	Good kernels	Good shells	Good nuts	Rotten kernels	Rotten shells	Rotten nuts	Total sample good plus rotten nuts
1	1	18.27	18.01	36.28	0.39	0.43	0.82	37.10
	2	16.13	15.75	31.88	1.08	1.25	2.33	34.21
	3	17.14	17.02	34.16	0.53	0.44	0.97	35.13
	4	18.10	17.86	35.96	0.72	0.74	1.46	37.42
	5	18.42	18.11	36.53	0.55	0.63	1.18	37.71
	6	17.28	16.88	34.16	0.30	0.49	0.79	34.95
	7	17.22	16.92	34.14	0.76	0.85	1.61	35.75
	8	16.76	16.48	33.24	0.69	0.76	1.45	34.69
	9	17.34	17.08	34.42	0.59	0.64	1.23	35.65
	10	17.32	17.14	34.46	0.27	0.24	0.51	34.97
2	1	17.26	13.91	31.17	1.29	1.45	2.74	33.91
	2	16.80	13.68	30.48	1.03	1.09	2.12	32.60
	3	16.98	13.77	30.75	0.98	1.10	2.08	32.83
	4	No data (see text)						
	5	17.21	13.91	31.12	1.13	1.28	2.41	33.53
	6	16.84	13.55	30.39	1.44	1.59	3.03	33.42
	7	17.26	13.85	31.11	1.34	1.55	2.89	34.00
	8	17.43	14.05	31.48	1.43	1.57	3.00	34.48
	9	17.02	13.78	30.80	1.18	1.29	2.47	33.27
	10	16.83	13.62	30.45	0.90	1.06	1.96	32.41
3	1	17.00	19.62	36.62	0.63	0.58	1.21	37.83
	2	16.74	19.28	36.02	0.90	0.93	1.83	37.85
	3	17.48	19.98	37.46	0.72	0.88	1.60	39.06
	4	17.80	20.51	38.31	0.53	0.49	1.02	39.33
	5	17.50	20.10	37.60	0.77	0.84	1.61	39.21
	6	17.26	19.84	37.10	0.92	0.99	1.91	39.01
	7	18.08	20.82	38.90	0.76	0.77	1.53	40.43
	8	19.20	22.04	41.24	0.81	0.89	1.70	42.94
	9	19.52	22.66	42.18	0.94	0.79	1.73	43.91
	10	No data						
4	1	17.20	18.91	36.11	0.78	0.73	1.51	37.62
	2	15.30	16.86	32.16	0.59	0.49	1.08	33.24
	3	No data						
	4	16.18	17.88	34.06	0.68	0.53	1.21	35.27
	5	16.80	18.40	35.20	0.49	0.48	0.97	36.17
	6	16.74	18.21	34.95	0.37	0.47	0.84	35.79
	7	16.66	18.23	34.89	0.79	0.83	1.62	36.51
	8	17.56	19.38	36.94	0.93	0.89	1.82	38.76

		Sample mass (kg)						
Lot	Sample	Good kernels	Good shells	Good nuts	Rotten kernels	Rotten shells	Rotten nuts	Total sample good plus rotten nuts
	9	17.96	19.92	37.88	0.68	0.52	1.20	39.08
	10	15.78	17.35	33.13	0.78	0.81	1.59	34.72
5	1	16.00	20.38	36.38	1.12	1.23	2.35	38.73
	2	16.02	20.39	36.41	0.85	0.91	1.76	38.17
	3	16.58	20.88	37.46	0.57	0.77	1.34	38.80
	4	17.14	21.68	38.82	0.73	0.88	1.61	40.43
	5	17.30	21.95	39.25	0.69	0.76	1.45	40.70
	6	16.68	21.09	37.77	0.44	0.52	0.96	38.73
	7	17.22	21.82	39.04	0.53	0.59	1.12	40.16
	8	15.20	19.24	34.44	0.33	0.37	0.70	35.14
	9	16.80	21.34	38.14	0.56	0.58	1.14	39.28
	10	17.46	22.17	39.63	0.63	0.67	1.30	40.93
6	1	18.86	18.22	37.08	0.72	0.74	1.46	38.54
7	1	17.50	15.77	33.27	0.64	0.77	1.41	34.68
8	1	17.50	15.99	33.49	0.56	0.68	1.24	34.73
9	1	17.18	16.42	33.60	0.78	0.84	1.62	35.22
10	1	17.14	16.24	33.38	0.49	0.77	1.26	34.64
11	1	18.02	16.67	34.69	0.56	0.69	1.25	35.94
12	1	15.40	15.04	30.44	0.48	0.65	1.13	31.57
All Lots	Sum of all samples	926.37	970.65	1897.02	40.35	43.78	84.13	1981.16
	Average	17.16	17.97	35.13	0.75	0.81	1.56	36.69
	% of total	46.76	48.99	95.75	2.04	2.21	4.25	100.00
	Maximum	19.52	22.66	42.18	1.44	1.59	3.03	43.91
	Minimum	15.20	13.55	30.39	0.27	0.24	0.51	31.57
	Median	17.21	18.06	34.92	0.72	0.77	1.46	36.06

Appendix 1b: CONFORCAST data – aflatoxin mass (µg) in Brazil nuts

		Aflatoxin mass (µg)						
Lot	Sample	Good kernels	Good shells	Good nuts	Rotten kernels	Rotten shells	Rotten nuts	Good and rotten nuts
1	1	4.75	102.38	107.13	154.66	363.18	517.84	624.96
	2	9.84	76.87	86.71	513.50	2911.33	3424.82	3511.53
	3	64.10	117.70	181.80	231.02	244.20	475.22	657.02
	4	11.58	149.11	160.69	263.45	366.71	630.16	790.85
	5	2.76	69.45	72.22	55.97	309.82	365.78	438.00
	6	66.70	94.67	161.37	136.37	224.45	360.82	522.19
	7	2.58	86.92	89.50	303.94	414.40	718.34	807.84
	8	2.68	73.86	76.54	251.75	117.72	369.47	446.01
	9	3.99	106.28	110.26	296.78	104.38	401.17	511.43
	10	104.44	88.18	192.61	118.18	94.00	212.18	404.79
2	1	0.35	57.30	57.65	271.67	222.23	493.90	551.55
	2	1.01	51.60	52.61	372.80	115.23	488.03	540.64
	3	1.70	26.22	27.92	663.03	99.33	762.36	790.28
	4	No data						
	5	7.57	38.87	46.44	274.70	17.60	292.30	338.74
	6	70.05	36.15	106.20	629.11	176.95	806.06	912.26
	7	278.40	86.35	364.76	498.91	347.08	845.98	1210.74
	8	38.87	32.31	71.18	256.44	68.34	324.78	395.97
	9	5.45	35.88	41.33	452.72	286.48	739.20	780.53
	10	1.01	24.15	25.16	239.47	67.85	307.32	332.48
3	1	119.85	53.02	172.87	157.40	145.66	303.06	475.93
	2	22.60	105.30	127.90	315.93	19.12	335.05	462.95
	3	4.37	92.88	97.25	189.61	47.03	236.64	333.89
	4	7.65	156.42	164.07	115.02	78.44	193.46	357.53
	5	4.55	201.08	205.63	302.62	168.56	471.18	676.81
	6	70.77	102.24	173.01	305.44	56.10	361.54	534.55
	7	4.16	52.48	56.64	159.13	51.51	210.64	267.28
	8	50.69	80.78	131.47	301.08	338.33	639.41	770.88
	9	33.38	148.52	181.90	184.14	136.28	320.42	502.32
	10	No data						
4	1	2.75	104.50	107.26	131.50	82.64	214.14	321.40
	2	3.83	118.90	122.73	177.56	46.07	223.63	346.36
	3	No data						
	4	7.44	97.01	104.45	202.78	58.24	261.02	365.47
	5	7.06	118.49	125.55	153.01	28.96	181.97	307.51
	6	8.37	141.00	149.37	110.72	84.38	195.10	344.47
	7	19.83	134.77	154.60	355.30	110.18	465.49	620.08
	8	47.24	128.55	175.79	304.67	36.92	341.59	517.37
	9	47.23	168.22	215.46	281.64	141.32	422.96	638.41
	10	227.55	188.64	416.19	237.39	150.78	388.17	804.36
5	1	3.84	90.06	93.90	357.34	103.63	460.96	554.86
	2	26.91	140.00	166.91	266.84	42.72	309.56	476.47

		Aflatoxin mass (µg)						
Lot	Sample	Good kernels	Good shells	Good nuts	Rotten kernels	Rotten shells	Rotten nuts	Good and rotten nuts
	3	4.31	126.82	131.13	205.83	48.54	254.37	385.50
	4	4.80	165.02	169.82	222.85	650.21	873.05	1042.87
	5	3.46	103.89	107.35	250.47	53.99	304.46	411.81
	6	1065.18	132.06	1197.25	155.57	130.11	285.69	1482.93
	7	15.50	82.96	98.45	195.43	67.02	262.45	360.91
	8	7.75	87.01	94.76	127.36	474.92	602.28	697.04
	9	6.38	104.00	110.39	205.82	205.27	411.09	521.48
	10	30.38	118.46	148.84	224.03	73.71	297.74	446.58
6	1	39.42	75.62	115.04	613.43	2.86	616.28	731.32
7	1	0.00	64.44	64.44	619.47	273.63	893.10	957.54
8	1	0.00	23.99	23.99	258.65	282.96	541.61	565.59
9	1	3.09	45.38	48.47	414.32	247.98	662.30	710.77
10	1	3.60	170.67	174.27	48.56	38.48	87.04	261.31
11	1	0.18	34.81	34.99	58.22	70.30	128.51	163.51
12	1	0.15	93.52	93.68	128.83	13.27	142.10	235.78
All Lots	Sum of all samples	2582.11	5205.75	7787.90	14322.39	11111.40	25433.79	33221.65
	Average	47.82	96.40	144.22	265.23	205.77	471.00	615.22
	% of total	7.77	15.67	23.44	43.11	33.45	76.56	100.00
	Maximum	1065.18	201.08	1197.25	663.03	2911.33	3424.82	3511.53
	Minimum	0.00	23.99	23.99	48.56	2.86	87.04	163.51
	Median	7.25	94.10	110.33	244.97	107.28	363.66	519.43

Appendix 1c: CONFORCAST data – aflatoxin concentrations (µg/kg) in Brazil nuts

		Aflatoxin concentration (µg/kg)						
Lot	Sample	Good kernels	Good shells	Good nuts	Rotten kernels	Rotten shells	Rotten nuts	Good and rotten nuts
1	1	0.26	5.69	2.95	396.56	844.60	631.51	16.85
	2	0.61	4.88	2.72	475.46	2329.06	1469.88	102.63
	3	3.74	6.92	5.32	435.88	555.01	489.92	18.70
	4	0.64	8.35	4.47	365.90	495.56	431.62	21.14
	5	0.15	3.83	1.98	101.76	491.77	309.99	11.61
	6	3.86	5.61	4.72	454.56	458.06	456.73	14.94
	7	0.15	5.14	2.62	399.92	487.53	446.17	22.60
	8	0.16	4.48	2.30	364.86	154.89	254.81	12.86
	9	0.23	6.22	3.20	503.02	163.10	326.15	14.35
	10	6.03	5.14	5.59	437.70	391.67	416.04	11.58
2	1	0.02	4.12	1.85	210.60	153.26	180.26	16.26
	2	0.06	3.77	1.73	361.94	105.72	230.20	16.59
	3	0.10	1.90	0.91	676.56	90.30	366.52	24.07
	4	No data						
	5	0.44	2.79	1.49	243.10	13.75	121.29	10.10
	6	4.16	2.67	3.49	436.88	111.29	266.03	27.30
	7	16.13	6.23	11.72	372.32	223.92	292.73	35.61
	8	2.23	2.30	2.26	179.33	43.53	108.26	11.48
	9	0.32	2.60	1.34	383.66	222.08	299.27	23.46
	10	0.06	1.77	0.83	266.08	64.01	156.80	10.26
3	1	7.05	2.70	4.72	249.84	251.14	250.46	12.58
	2	1.35	5.46	3.55	351.03	20.56	183.09	12.23
	3	0.25	4.65	2.60	263.35	53.44	147.90	8.55
	4	0.43	7.62	4.28	217.01	160.09	189.67	9.09
	5	0.26	10.01	5.47	393.01	200.67	292.66	17.26
	6	4.10	5.15	4.66	332.00	56.67	189.29	13.70
	7	0.23	2.52	1.46	209.38	66.90	137.67	6.61
	8	2.64	3.67	3.19	371.70	380.15	376.12	17.95
	9	1.71	6.56	4.31	195.89	172.51	185.21	11.44
	10	No data						
4	1	0.16	5.53	2.97	168.59	113.21	141.82	8.54
	2	0.25	7.05	3.82	300.95	94.02	207.07	10.42
	3	No data						
	4	0.46	5.43	3.07	298.20	109.89	215.72	10.36
	5	0.42	6.44	3.57	312.26	60.33	187.59	8.50
	6	0.50	7.74	4.27	299.24	179.54	232.27	9.62
	7	1.19	7.39	4.43	449.75	132.75	287.34	16.99
	8	2.69	6.63	4.76	327.60	41.48	187.68	13.35
	9	2.63	8.45	5.69	414.18	271.76	352.46	16.34
	10	14.42	10.87	12.56	304.35	186.15	244.13	23.17
5	1	0.24	4.42	2.58	319.05	84.25	196.15	14.33
	2	1.68	6.87	4.58	313.93	46.94	175.88	12.48

		Aflatoxin concentration (µg/kg)						
Lot	Sample	Good kernels	Good shells	Good nuts	Rotten kernels	Rotten shells	Rotten nuts	Good and rotten nuts
	3	0.26	6.07	3.50	361.11	63.04	189.83	9.94
	4	0.28	7.61	4.37	305.27	738.87	542.27	25.79
	5	0.20	4.73	2.73	363.00	71.04	209.97	10.12
	6	63.86	6.26	31.70	353.57	250.22	297.59	38.29
	7	0.90	3.80	2.52	368.74	113.59	234.33	8.99
	8	0.51	4.52	2.75	385.93	1283.57	860.40	19.84
	9	0.38	4.87	2.89	367.53	353.92	360.61	13.28
	10	1.74	5.34	3.76	355.61	110.01	229.03	10.91
6	1	2.09	4.15	3.10	851.98	3.86	422.11	18.97
7	1	0.00	4.08	1.94	967.92	355.36	633.40	27.61
8	1	0.00	2.76	0.72	461.87	416.12	436.78	16.29
9	1	0.18	10.51	1.44	531.18	295.21	408.83	20.18
10	1	0.21	2.09	5.22	99.10	49.97	69.08	7.54
11	1	0.01	2.09	1.01	103.96	101.88	102.81	4.55
12	1	0.01	6.22	3.08	268.40	20.41	125.75	7.47
All lots	Sum of all samples	2.79	5.36	4.11	354.95	253.80	302.32	16.77
	Average	2.83	5.27	4.05	357.46	264.97	310.32	16.96
	% of total	16.62	31.98	24.48	2116.75	1513.53	1802.84	100.00
	Maximum	63.86	10.87	31.70	967.92	2329.06	1469.88	102.63
	Minimum	0.00	1.77	0.72	99.10	3.86	69.08	4.55
	Median	0.43	5.15	3.15	358.36	154.08	247.30	13.53

APPENDIX 2

Statistical assessment to derive the relationship between concentration of aflatoxins in nuts and the concentration in the kernel (the conversion factor)

Linear regression of log transformed concentration on nuts against the proportion in the kernel was undertaken for all nuts, rotten nuts and good nuts. Similarly the relation between log transformed concentration and the proportion of rotten nuts in the sample was examined. A single concentration-independent factor for converting between nut-concentration and kernel-concentration was found to be consistent with the observed results. An analysis of variance and bootstrap re-sampling (where values are repeatedly re-sampled with replacement to estimate the size of the uncertainty associated with statistics such as the mean) was used to estimate the size of within and between lot variation in the value of the factor, and the size of the uncertainty associated with the mean value of the factor.

Information to allow the utility of the factor to be assessed was provided in the form of estimates of the uncertainty associated with kernel concentration estimates where a) kernel concentration was measured directly in shelled nuts 2) kernel concentration was estimated using a factor derived from the relations observed in this study; 3) kernel concentration was estimated by applying a factor of '2' to the measured concentration in whole nuts.

Variation and uncertainty in the relation between the concentration of aflatoxins in in-shell nuts and the concentration of aflatoxins in kernels

The relation between the concentration of aflatoxin in a body of in-shell nuts and the concentration of aflatoxin in the kernels of those nuts can be described by a conversion factor F_B given by:

$$F_B = \frac{C_k}{C_i}$$

Where C_k is the concentration of aflatoxins in the kernels and C_i is the concentration of aflatoxins in the in-shell nuts.

If we assume, based on the observations from 12 lots, that a single concentration-independent factor F describes the relation between the concentration of aflatoxins in in-shell nuts and kernels across all lots, then an observed factor F_B is given by

$$F_B = F + L + S + e$$

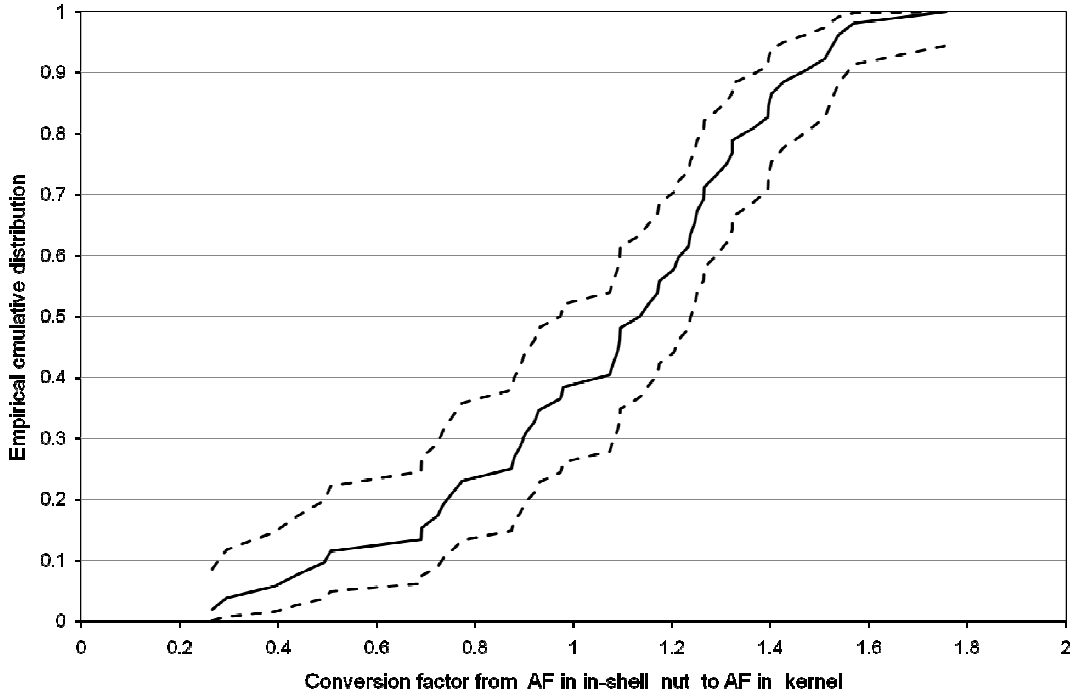
Where L is bias associated with a lot, S is the bias associated with a sample from the lot and e is the error associated with the estimate of F_B. The variation in the observed values of F_B can be modelled as

$$s_T^2 = s_L^2 + s_S^2 + s_e^2$$

Where s_T is the total standard deviation, s_L is the between-lot contribution, s_s is the contribution made by sampling and s_e is the within-sample standard deviation.

54 estimates of F_B were calculated using the CONFORCAST data (10 each from replicate samples taken from two lots, 9 from 3 lots and seven from single samples taken from seven lots). The average of estimate of F_B was 1.07. The 10th percentile for a population of lots was estimated to lie between 0.28 and 0.73; and the 90th percentile was estimated to lie between 1.37 and 1.56 (Figure A). The distribution of factors was consistent with both normal and beta distributions.

Figure A: Distribution of estimates of conversion factor for samples of at least 30 kg



——— Estimated distribution of 30 kg samples

– – 95% confidence interval for estimate

AF = aflatoxin

An analysis of variance yielded the following estimates

s_L	0.194
$\sqrt{s_s^2 + s_e^2}$	0.294
s_T	0.352

The effect of between-lot and between-sample variation was also estimated using bootstrap sampling where the data is resampled with replacement to gain a number of estimates of the

conversion factor whose range reflects the uncertainty associated with the value estimated from the original data. Bootstrap samples from within-lots were used to estimate the value of the correction factor for each lot. Similarly a bootstrap sample from between lots (where each within lot estimate was based on a new bootstrap sample) was used to estimate the value of the conversion factor F and its uncertainty. Estimates of the lot conversion factors, for the five lots which had yielded 10 samples, lay between 0.566 and 1.406. The estimated mean conversion factor F was 1.052 with a 95% confidence interval from 0.859 to 1.246 (standard deviation 0.098).

The uncertainty associated with analytical results and concentration estimates derived from analytical results

The estimated value of the factor that converts measurements of the concentration of aflatoxin in in-shell nuts to the concentration in the kernel is 1.052 for both the mean factor across lots (F) and the factor for an individual sample taken from a lot (F_B). However the size of the uncertainty associated with F and F_B is different. For example, an estimate F is used to convert the mean concentration of aflatoxins in in-shell nuts ($\overline{C_i}$) to the mean concentration in kernels ($\overline{C_k}$) (e.g. for use in an assessment of mean exposure). The relative standard uncertainty (RSU) associated with the factor is estimated to be 0.0932 (0.098/1.052) and the relative standard uncertainty associated with an estimate of the mean concentration of aflatoxins in the kernel is given by:

$$RSU_{\overline{C_k}} = \sqrt{RSU_{\overline{F}}^2 + RSU_{\overline{C_i}}^2}$$

Hence, -

$$RSU_{\overline{C_k}} = \sqrt{0.0932^2 + RSU_{\overline{C_i}}^2}$$

Where -

$$\overline{C_k} = F\overline{C_i}$$

and

$RSU_{\overline{C_i}}$ is the relative standard uncertainty associated with the estimated mean concentration of aflatoxins in in-shell nuts

$RSU_{\overline{F}}$ is the relative standard uncertainty associated with the mean conversion factor across lots

Where an estimate of the factor F_B is used to convert an individual result based on the measurement of aflatoxins in a 30 kg sample of in-shell nuts (from C_k to C_i) (e.g. for control), the relative standard uncertainty associated with the factor is 0.335 (0.352/1.05) and the relative standard uncertainty associated with the concentration of aflatoxin in the kernels (RSU_{C_K}) is given by:

$$RSU_{C_K} = \sqrt{RSU_{F_B}^2 + RSU_{C_i}^2}$$

Hence, -

$$RSU_{C_K} = \sqrt{0.335^2 + RSU_{C_i}^2}$$

Where -

$C_k = F_E C_i$

and

$RSU_{C_i}^2$ is the relative standard uncertainty associated with the measured concentration of aflatoxins in a 30 kg sample of in-shell nuts

$RSU_{F_B}^2$ is the relative standard uncertainty associated with the value of the conversion factor for a 30 kg sample taken from a lot

If the relative standard uncertainty associated with analytical results is consistent with the modified Horwitz equation then the relative standard uncertainty associated with the concentration measured in in-shell nuts (RSU_{C_i}) is equal to 0.22 and the relative standard uncertainty associated with the estimated concentration of aflatoxins in the kernels in the sample is equal to 0.40.

For example, if the concentration in kernels is measured directly and the analysis of a 30 kg sample of shelled kernels for total aflatoxin yields a result of 40 µg/kg, then:

C_k=40 µg/kg

$$RSU_{C_K} = 0.22$$

C_k = 40 ± 17.6 µg/kg (using coverage factor = 2) -

the 95% confidence interval for the concentration of aflatoxins in the sample of shelled kernels is - 22.4 to 57.6 µg/kg. -

However, if a similar result were produced by the analysis of in-shell nuts then: -

C_i = 40 µg/kg -

C_k = 40 × 1.052 -

$$RSU_{C_K} = \sqrt{0.335^2 + 0.22_{C_i}^2} = 0.40$$

C_k = 42.0 ± 33.6 µg/kg (using coverage factor = 2) -

the 95% confidence interval for the concentration of aflatoxins in the kernels of the sample is 8.4 to - 75.7 µg/kg. This estimate includes sampling uncertainty about the value of the factor that converts

between in-shell and kernel concentrations, but does not include sampling uncertainty about the in-lot mean in-shell concentration. i.e. the concentration estimate and its uncertainty applies to the kernels in the sample

Hence, the measurement uncertainty associated with an estimate of the concentration of aflatoxins in kernels based on measurements of aflatoxins in in-shell nuts is approximately double the size of the uncertainty associated with the direct measurement of aflatoxin in shelled kernels (95% confidence interval of ± 44% for direct measurement, ± 80% estimates derived from measurement of in-shell nuts). The actual values will vary depending on the measurement uncertainty estimate calculated for each laboratory, because it is unlikely that the Horwitz value will be used in all cases.

Using the calculated conversion factor of 1.05, if in-shell measurements are used to enforce a regulatory limit for concentration in kernels, it is necessary to measure total aflatoxin levels in excess of 50 µg/kg in a sample of in-shell Brazil nuts to have 95% confidence that the kernels in the sample exceeded the maximum limit of 10 µg/kg (Regulation (EC) No. 401/2006, Regulation (EU) No. 165/2010).

STATISTICAL OUTPUTS

Appendix 2a. Relation between concentration in good nuts and proportion in the kernel

```
GenStat Release 13.1 ( PC/Windows XP) 28 March 2011 11:13:59
Copyright 2010, VSN International Ltd.
Registered to: Fera

                    ____________________________________

                    GenStat Thirteenth Edition
                    GenStat Procedure Library Release PL21.1
                    ____________________________________

  1  %CD 'C:/Documents and Settings/rmacarth/My Documents'
  2  "Data taken from unsaved spreadsheet: New Data;1"
  3  DELETE [REDEFINE=yes] _stitle_: TEXT _stitle_
  4  READ [PRINT=*; SETNVALUES=yes] _stitle_
  7  PRINT [IPRINT=*] _stitle_; JUST=left

Data imported from Clipboard
 on: 28-Mar-2011 11:14:10

  8  DELETE [REDEFINE=yes] LOT,proportion_AF_in_kernel,conc_in_good_nuts,\
  9  Ln_Conc_in_good_nuts
 10  UNITS [NVALUES=*]
 11  VARIATE [NVALUES=57] LOT
 12  READ LOT

      Identifier      Minimum         Mean      Maximum       Values      Missing
             LOT        1.000        3.737        12.00           57            0  Skew

 15  VARIATE [NVALUES=57] proportion_AF_in_kernel
 16  READ proportion_AF_in_kernel

                  Identifier      Minimum         Mean      Maximum       Values      Missing
proportion_AF_in_kernel          0.0000       0.1782       0.8897           57            3
Skew

 31  VARIATE [NVALUES=57] conc_in_good_nuts
 32  READ conc_in_good_nuts

          Identifier      Minimum         Mean      Maximum       Values      Missing
conc_in_good_nuts          0.7200        4.051        31.70           57            3  Skew

 37  VARIATE [NVALUES=57] Ln_Conc_in_good_nuts
 38  READ Ln_Conc_in_good_nuts

              Identifier      Minimum         Mean      Maximum       Values      Missing
Ln_Conc_in_good_nuts         -0.3285        1.147        3.456           57            3
```

```
  53
  54  %PostMessage 1129; 0; 23389032 "Sheet Update Completed"
  55  "Simple Linear Regression"
  56  MODEL Ln_Conc_in_good_nuts
  57  TERMS proportion_AF_in_kernel
  58  FIT [PRINT=model,summary,estimates; CONSTANT=estimate; FPROB=yes;
TPROB=yes] proportion_AF_in_kernel
```

Regression analysis

Response variate: Ln_Conc_in_good_nuts
Fitted terms: Constant, proportion_AF_in_kernel

Summary of analysis

Source	d.f.	s.s.	m.s.	v.r.	F pr.
Regression	1	8.68	8.6753	31.82	<.001
Residual	52	14.18	0.2726		
Total	53	22.85	0.4312		

Percentage variance accounted for 36.8 -
Standard error of observations is estimated to be 0.522. -

Message: the following units have high leverage.

Unit	Response	Leverage
16	1.250	0.107
17	2.461	0.150
21	1.552	0.120
46	3.456	0.213

Estimates of parameters

Parameter	estimate	s.e.	t(52)	t pr.
Constant	0.8214	0.0915	8.98	<.001
proportion_AF_in_kernel	1.825	0.324	5.64	<.001

```
  59  RCHECK [RMETHOD=deviance] residual; composite
  60  RGRAPH [CIPLOT=yes]
  61  PREDICT [PRINT=description,predictions,se; COMBINATIONS=estimable;
SCOPE=new] CLASSIFY=proportion_AF_in_kernel;\
  62
LEVELS=!(0,0.05,0.1,0.05,0.1,0.15,0.2,0.25,0.3,0.35,0.4,0.45,0.5,0.55,0.6,0
.65,0.7,\
  63  0.75,0.8,0.85,0.9,0.95,1)
```

Predictions from regression model

These predictions are estimated mean values.

The standard errors are appropriate for interpretation of the predictions as forecasts of new observations rather than as summaries of the data.

Response variate: Ln_Conc_in_good_nuts

proportion_AF_in_kernel	Prediction	s.e.
0.00	0.821	0.5301
0.05	0.913	0.5286
0.10	1.004	0.5276
0.05	0.913	0.5286
0.10	1.004	0.5276
0.15	1.095	0.5270
0.20	1.186	0.5270
0.25	1.278	0.5275
0.30	1.369	0.5284
0.35	1.460	0.5299
0.40	1.551	0.5318
0.45	1.643	0.5342
0.50	1.734	0.5371
0.55	1.825	0.5405
0.60	1.916	0.5443
0.65	2.008	0.5486
0.70	2.099	0.5533
0.75	2.190	0.5585
0.80	2.281	0.5640
0.85	2.373	0.5700
0.90	2.464	0.5764
0.95	2.555	0.5831
1.00	2.646	0.5902

Appendix 2b. Relation between concentration in rotten nuts and proportion in the kernel

```
 70  DELETE [REDEFINE=yes] LOT,conc_in_rotten_nut,log_conc_in_rotten_nut
 71  UNITS [NVALUES=*]
 72  FACTOR [MODIFY=yes; NVALUES=57; LEVELS=12; REFERENCE=1] LOT
 73  READ LOT; FREPRESENTATION=ordinal
```

Identifier	Values	Missing	Levels -
LOT	57	0	12 -

```
 76  VARIATE [NVALUES=57] proportion_AF_in_kernel
 77  READ proportion_AF_in_kernel
```

Identifier	Minimum	Mean	Maximum	Values	Missing
proportion_AF_in_kernel	0.1499	0.6411	0.9954	57	3

```
 92  VARIATE [NVALUES=57] conc_in_rotten_nut
 93  READ conc_in_rotten_nut
```

Identifier	Minimum	Mean	Maximum	Values	Missing	
conc_in_rotten_nut	69.08	310.3	1470	57	3	Skew

```
100  VARIATE [NVALUES=57] log_conc_in_rotten_nut
101  READ log_conc_in_rotten_nut
```

Identifier	Minimum	Mean	Maximum	Values	Missing
log_conc_in_rotten_nut	4.235	5.570	7.293	57	3

```
116
117  %PostMessage 1129; 0; 25052136 "Sheet Update Completed"
118  "Simple Linear Regression"
119  MODEL log_conc_in_rotten_nut
120  TERMS proportion_AF_in_kernel
121  FIT [PRINT=model,summary,estimates; CONSTANT=estimate; FPROB=yes;
TPROB=yes] proportion_AF_in_kernel
```

Regression analysis

Response variate: log_conc_in_rotten_nut -
Fitted terms: Constant, proportion_AF_in_kernel -

Summary of analysis

Source	d.f.	s.s.	m.s.	v.r.	F pr. -
Regression	1	5.45	5.4535	25.65	<.001 -
Residual	52	11.05	0.2126 -		
Total	53	16.51	0.3115 -		

Percentage variance accounted for 31.7 -
Standard error of observations is estimated to be 0.461. -

Message: the following units have large standardized residuals.

Unit	Response	Residual
55	4.235	-3.21

56	4.633	-2.72

Message: the following units have high leverage.

Unit	Response	Leverage
2	7.293	0.128
5	5.737	0.127
48	6.757	0.102

Estimates of parameters

Parameter	estimate	s.e.	t(52)	t pr.
Constant	6.579	0.209	31.52	<.001
proportion_AF_in_kernel	-1.573	0.310	-5.07	<.001

```
 122  RCHECK [RMETHOD=deviance] residual; composite
 123  RGRAPH [CIPLOT=yes]
 124  PREDICT [PRINT=description,predictions,se; COMBINATIONS=estimable;
SCOPE=new] CLASSIFY=proportion_AF_in_kernel;\
 125
LEVELS=!(0,0.05,0.1,0.05,0.1,0.15,0.2,0.25,0.3,0.35,0.4,0.45,0.5,0.55,0.6,0
.65,0.7,\
 126  0.75,0.8,0.85,0.9,0.95,1)
```

Predictions from regression model

These predictions are estimated mean values.

The standard errors are appropriate for interpretation of the predictions as forecasts of new observations rather than as summaries of the data.

Response variate: log_conc_in_rotten_nut

proportion_AF_in_kernel	Prediction	s.e.
0.00	6.579	0.5061
0.05	6.500	0.5002
0.10	6.421	0.4947
0.05	6.500	0.5002
0.10	6.421	0.4947
0.15	6.343	0.4897
0.20	6.264	0.4850
0.25	6.185	0.4809
0.30	6.107	0.4772
0.35	6.028	0.4740
0.40	5.949	0.4713
0.45	5.871	0.4691
0.50	5.792	0.4674
0.55	5.714	0.4662
0.60	5.635	0.4655
0.65	5.556	0.4653
0.70	5.478	0.4657
0.75	5.399	0.4665
0.80	5.320	0.4679
0.85	5.242	0.4698
0.90	5.163	0.4722

0.95	5.085	0.4751
1.00	5.006	0.4785

Appendix 2c. Relation between concentration in all nuts and proportion in the kernel

```
133  DELETE [REDEFINE=yes]
LOT,proportion_in_all_kernels,logconc_in_all_nuts
 134  UNITS [NVALUES=*]
 135  VARIATE [NVALUES=57] LOT
 136  READ LOT
```

Identifier	Minimum	Mean	Maximum	Values	Missing	
LOT	1.000	3.737	12.00	57	0	Skew

```
 139  VARIATE [NVALUES=57] proportion_in_all_kernels
 140  READ proportion_in_all_kernels
```

Identifier	Minimum	Mean	Maximum	Values	Missing
proportion_in_all_kernels	0.1341	0.5261	0.8927	57	4

```
 155  VARIATE [NVALUES=57] logconc_in_all_nuts
 156  READ logconc_in_all_nuts
```

Identifier	Minimum	Mean	Maximum	Values	Missing
logconc_in_all_nuts	1.515	2.674	4.631	57	3

```
 171
 172  %PostMessage 1129; 0; 39243800 "Sheet Update Completed"
 173  "Simple Linear Regression"
 174  MODEL logconc_in_all_nuts
 175  TERMS proportion_in_all_kernels
 176  FIT [PRINT=model,summary,estimates; CONSTANT=estimate; FPROB=yes;
TPROB=yes] proportion_in_all_kernels
```

Regression analysis

Response variate: logconc_in_all_nuts -
Fitted terms: Constant, proportion_in_all_kernels -

Summary of analysis

Source	d.f.	s.s.	m.s.	v.r.	F pr.
Regression	1	0.07	0.0699	0.28	0.599
Residual	51	12.70	0.2490		
Total	52	12.77	0.2455		

Residual variance exceeds variance of response variate.
Standard error of observations is estimated to be 0.499.

Message: the following units have large standardized residuals.

Unit	Response	Residual
2	4.631	4.02
56	1.515	-2.40

Message: the following units have high leverage.

Unit	Response	Leverage
2	4.631	0.107

5	2.452	0.115
51	2.943	0.103

Estimates of parameters

Parameter	estimate	s.e.	t(51)	t pr.
Constant	2.765	0.218	12.68	<.001
proportion_in_all_kernels	-0.209	0.394	-0.53	0.599

```
 177  RCHECK [RMETHOD=deviance] residual; composite
 178  RGRAPH [CIPLOT=yes]
 179  PREDICT [PRINT=description,predictions,se; COMBINATIONS=estimable;
SCOPE=new] CLASSIFY=proportion_in_all_kernels;\
 180
LEVELS=!(0,0.05,0.1,0.05,0.1,0.15,0.2,0.25,0.3,0.35,0.4,0.45,0.5,0.55,0.6,0
.65,0.7,\
 181  0.75,0.8,0.85,0.9,0.95,1)
```

Predictions from regression model

These predictions are estimated mean values.

The standard errors are appropriate for interpretation of the predictions as forecasts of new observations rather than as summaries of the data.

Response variate: logconc_in_all_nuts

proportion_in_all_kernels	Prediction	s.e.
0.00	2.765	0.5446
0.05	2.755	0.5374
0.10	2.744	0.5309
0.05	2.755	0.5374
0.10	2.744	0.5309
0.15	2.734	0.5250
0.20	2.723	0.5198
0.25	2.713	0.5153
0.30	2.703	0.5115
0.35	2.692	0.5084
0.40	2.682	0.5061
0.45	2.671	0.5046
0.50	2.661	0.5038
0.55	2.650	0.5038
0.60	2.640	0.5045
0.65	2.630	0.5060
0.70	2.619	0.5083
0.75	2.609	0.5113
0.80	2.598	0.5151
0.85	2.588	0.5196
0.90	2.577	0.5247
0.95	2.567	0.5306
1.00	2.557	0.5371

Appendix 2d. Relation between the concentration of aflatoxins in all nuts and the proportion of rotten nuts in the sample

```
188  DELETE [REDEFINE=yes] LOT,proportion_of_lot_that_is_rotten
 189  UNITS [NVALUES=*]
 190  FACTOR [MODIFY=yes; NVALUES=57; LEVELS=12; REFERENCE=1] LOT
 191  READ LOT; FREPRESENTATION=ordinal
```

Identifier	Values	Missing	Levels -
LOT	57	0	12 -

```
 194  VARIATE [NVALUES=57] proportion_of_lot_that_is_rotten
 195  READ proportion_of_lot_that_is_rotten
```

Identifier	Minimum	Mean	Maximum	Values	Missing
proportion_of_lot_that_is_rotten	0.02860	0.08254	0.1525	57	3

```
 210  VARIATE [NVALUES=57] logconc_in_all_nuts
 211  READ logconc_in_all_nuts
```

Identifier	Minimum	Mean	Maximum	Values	Missing
logconc_in_all_nuts	1.515	2.674	4.631	57	3

```
 226
 227  %PostMessage 1129; 0; 39661736 "Sheet Update Completed"
 228  "Simple Linear Regression"
 229  MODEL logconc_in_all_nuts
 230  TERMS proportion_of_lot_that_is_rotten
 231  FIT [PRINT=model,summary,estimates; CONSTANT=estimate; FPROB=yes;
TPROB=yes] proportion_of_lot_that_is_rotten
```

Regression analysis

Response variate: logconc_in_all_nuts -
Fitted terms: Constant, proportion_of_lot_that_is_rotten -

Summary of analysis

Source	d.f.	s.s.	m.s.	v.r.	F pr. -
Regression	1	1.60	1.6028	6.87	0.011 -
Residual	52	12.13	0.2332 -		
Total	53	13.73	0.2590 -		

Percentage variance accounted for 10.0 -
Standard error of observations is estimated to be 0.483. -

Message: the following units have large standardized residuals.

Unit	Response	Residual
2	4.631	3.61
46	3.645	2.41

Message: the following units have high leverage.

Unit	Response	Leverage
10	2.449	0.087

11	2.789	0.088
16	3.307	0.134
17	3.573	0.106
18	2.441	0.116

Estimates of parameters

Parameter	estimate	s.e.	t(52)	t pr.
Constant	2.167	0.204	10.61	<.001
proportion_of_lot_that_is_rotten	6.14	2.34	2.62	0.011

```
 232  RCHECK [RMETHOD=deviance] residual; composite
 233  RGRAPH [CIPLOT=yes]
 234  PREDICT [PRINT=description,predictions,se; COMBINATIONS=estimable;
SCOPE=new] CLASSIFY=proportion_of_lot_that_is_rotten;\
 235
LEVELS=!(0.04,0.045,0.05,0.055,0.06,0.065,0.07,0.075,0.08,0.085,0.09,0.095,
0.1,0.105,\
 236  0.11,0.115,0.12,0.125,0.13,0.135,0.14,0.145,0.15,0.155)
```

Predictions from regression model

These predictions are estimated mean values.

The standard errors are appropriate for interpretation of the predictions as forecasts of new observations rather than as summaries of the data.

Response variate: logconc_in_all_nuts

proportion_of_lot_that_is_rotten	Prediction	s.e.
0.040	2.413	0.4974
0.045	2.443	0.4952
0.050	2.474	0.4933
0.055	2.505	0.4916
0.060	2.535	0.4902
0.065	2.566	0.4891
0.070	2.597	0.4882
0.075	2.628	0.4877
0.080	2.658	0.4874
0.085	2.689	0.4874
0.090	2.720	0.4877
0.095	2.750	0.4882
0.100	2.781	0.4891
0.105	2.812	0.4902
0.110	2.842	0.4916
0.115	2.873	0.4933
0.120	2.904	0.4952
0.125	2.935	0.4974
0.130	2.965	0.4999
0.135	2.996	0.5026
0.140	3.027	0.5056
0.145	3.057	0.5089
0.150	3.088	0.5123
0.155	3.119	0.5161

Appendix 2e. Anova of concentration in lots

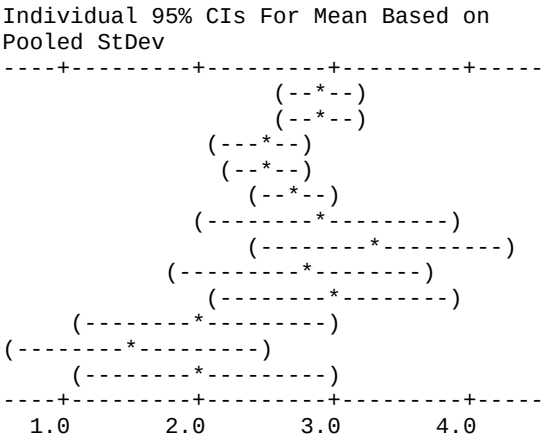
```
————— 29/03/2011 14:15:28 ————————————————————————

Welcome to Minitab, press F1 for help.
```

One-way ANOVA: logconc in all nuts versus LOT

```
Source  DF      SS     MS     F      P
LOT     11   4.573  0.416  1.91  0.066
Error   42   9.156  0.218
Total   53  13.729

S = 0.4669   R-Sq = 33.31%   R-Sq(adj) = 15.84%
```



Level	N	Mean	StDev
1.000	10	2.9376	0.6392
2.000	9	2.8772	0.4557
3.000	9	2.4520	0.3268
4.000	9	2.5101	0.3500
5.000	10	2.6852	0.4694
6.000	1	2.9429	*
7.000	1	3.3182	*
8.000	1	2.7906	*
9.000	1	3.0047	*
10.000	1	2.0202	*
11.000	1	1.5151	*
12.000	1	2.0109	*

```
Pooled StDev = 0.4669
```

Anova of conversion factors

Identifier	Values	Missing	Levels
lot	57	0	12

```
                44
 45  %PostMessage 1129; 0; 24956952 "Sheet Update Completed"
 46  "One-way design"
 47  DELETE [REDEFINE=yes] _ibalance
 48  A2WAY [PRINT=aovtable,information,means; TREATMENTS=lot; FPROB=yes; PSE=diff; PLOT=fitt,\
 49  norm,half,hist; EXIT=_ibalance] correction_for_all; SAVE=_a2save
```

Analysis of variance

Variate: correction_for_all

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
lot	11	2.64517	0.24047	2.78	0.008
Residual	41	3.54056	0.08636		
Total	52	6.18573			

Information summary

All terms orthogonal, none aliased.

Message: the following units have large residuals.

units 41	-0.613	approx. s.e.	0.258
units 44	-0.668	approx. s.e.	0.258

Note: 4 missing units have been omitted from the analysis.

Tables of means

Grand mean 1.0711

lot	1	2	3	4	5
mean	0.7578	1.2845	1.1673	1.1076	1.1067
rep.	10	9	9	9	9
lot	6	7	8	9	10
mean	1.7573	1.237	0.8794	1.1515	0.3922
rep.	1	1	1	1	1
lot	11	12			
mean	0.6908	1.0876			
rep.	1	1			

Minimum standard error of difference	0.135

Average standard error of difference	0.3171
Maximum standard error of difference	0.4156

correction_for_all