



## MONTE CARLO MODELING FOR AFLATOXIN B<sub>1</sub> DISTRIBUTION IN PISTACHIO SAMPLES: A PREREQUISITE FOR SAMPLING PLAN VALIDATION

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### ABSTRACT

*AFB<sub>1</sub> is the main aflatoxin found in pistachios, and as it is dangerous for health, the European Union (EU) has set a maximum level at 8 µg/kg in pistachios in 10 kg samples. Given that efficient sampling plans must be used in order to determine this contaminant level accurately, a mathematical validation of sampling plans has to be achieved with to Operating Characteristics curves. However, a prerequisite before drawing these curves is to assess the contaminant distribution within the food lot as well as to determine the variability between samples taken from the food lot. This is difficult to achieve for AFB<sub>1</sub> in pistachios, as the contamination incidence rate is low, and concentrations differ greatly between food items. More precisely, the contaminant is heterogeneously distributed. Contrarily to current contaminant distribution assessment methods used on a regulatory basis and which are based directly on variability between samples, the method developed relied first on variability between individual pistachios, before computing the variability between samples, thus triggering more reliable results. This work assessed the distribution and incidence of aflatoxin in pistachio nuts, thanks to a Monte Carlo simulation, with pistachios split into categories, which were early split sound kernel, cracked sound kernel, and mouldy pistachios. A 30 kg sample size of Iranian pistachios for export to Europe was considered. Differences between good and bad lots were taken into account with lots split into 3 classes for mean contamination levels in export packages. Simulation results were in good accordance with observed contamination levels in Iran.*

**Keywords:** AFB<sub>1</sub>, Cracked, Early split, Heterogeneous distribution, Mouldy, Mycotoxin.

### 1. INTRODUCTION

Efficient sampling plans must be used in order to determine food contaminant levels accurately. Indeed, a representative sampling plan enables to take samples from a food lot, following a given protocol, with a relatively low risk of misestimating the true mean concentration of the food lot after analysis of the food samples. A mathematical validation of sampling plans can be achieved thanks to Operating Characteristics curves (Spanjer, 2005). However, a prerequisite before drawing these curves is to assess the contaminant distribution within the food lot as well as to determine the variability between samples taken from the food lot (Whitaker *et al.*, 1972; 2007a; 2007b) The cases for which accurate contaminant distributions are

the most difficult to obtain are the ones with contaminants distributed heterogeneously in a food lot (Miraglia *et al.*, 2008). In such cases, the contamination incidence rate is low, and concentrations differ greatly between food items. Such a heterogeneity problem can be observed for mycotoxins produced by moulds on granular food items like cereals, tree nuts ... Among these mycotoxins, aflatoxins, which are mainly produced by two species of *Aspergillus* fungi: *A. flavus* and *A. Parasiticus* (Yu *et al.*, 1995), have genotoxic carcinogenic effects, with no threshold. They contaminate pistachios in the orchard, when *Aspergillus* spores, carried by insects or wind get into contact mainly with pistachios having their hull ruptured (Doster and Michailides, 1994). Indeed, the hull protects in-shell pistachios when it is not split. Finally, after industrial processing, contamination does not increase in case of proper storage condition (Miller, 1995). One type of aflatoxin: aflatoxin B1 (AFB1) is the main aflatoxin found in pistachios, as well as the most toxic. Due to its threat for human health, the European Union (EU) has set a maximum level at 8 µg/kg in pistachios (Regulation, 2010) in 10 kg samples (Regulation, 2010). Depending on harvest year, aflatoxin can cause a high rejection rate of Iranian pistachios at the point of entry into the EU. Rejections are reported through the RASFF (Rapid Alert System for Food and Feed) and cause great financial losses. A ban on Iranian pistachio imports, due to high levels of aflatoxin, occurred in September 1997. The embargo was lifted three months later following assurances that the quality of Iranian pistachio would be improved. Aflatoxin level in pistachio consignments is difficult to assess, knowing that there is a high contamination variability between individual pistachios and thus, between samples taken from the same lot. Per example, it has been estimated that only one pistachio out of 10000 to 1000000 might be highly contaminated (Schatzki and Pan, 1996.), or one out of 25000 nuts (Sommer *et al.*, 1986). The sampling plan to be used has been validated on the basis of Whitaker's method for the determination of aflatoxin distribution and variability in tree nuts (Codex). However, this evaluation method has some limitations due to the fact that it could be misleading, as it is mainly based on experimental contamination variability data for a number of samples taken from the same lot, without considering the proportion of pistachios that are the most at risk to be contaminated. Moreover, it is derived from the analysis results of a relatively limited number of samples in comparison to the low incidence of contamination for obtaining the regression equation of sampling variance as function of lot mean concentration. For this reason, this work focuses on the development of a new method, based upon the Monte Carlo technique, for distribution and variability assessment for AFB1 in pistachios, considering prior knowledge of the contamination pattern. The Monte Carlo technique is used to estimate the effect of variability on the outputs of models which use a variety of input parameters with variability. It can be used to generate probability density functions of contamination levels obtained thanks to the combination of various distributions. The objective of this study is to model concentration distribution in 30 kg samples, based on specific categories of pistachios having particular visual defects and often cited in the literature as having a high probability of being contaminated. Indeed, the proportion of these categories comprised in samples, as well as their contamination probability and contamination level distribution have an impact on the variability between 30 kg samples taken from the same lot. Pistachio categories considered are early split sound kernel, cracked sound kernel and mouldy pistachios. Differences

between good and bad lots are taken into account with lots split into 3 classes for mean contamination levels in samples.

## 2. DATA USED AND METHODS

### 2.1. Pistachio Categories Choice

In the orchard, when pistachios ripen, their shell splits. This is a desirable feature resulting in high value pistachios. Nevertheless at harvest stage, some pistachios, having specific characteristics, are likely to be highly contaminated by AFB1. These distinctive features are various forms of hull rupture, or kernel necrosis observed on pistachio sections or even the presence of a fungal colony. These suspicious nuts are categorized in early split sound kernel, cracked sound kernel and mouldy pistachios as described below. For the two first categories, only pistachios having sound kernels, it says non mouldy, were considered for the simulation process. It enabled to make modeling more convenient. When the hull surrounding the shell is intact at harvest, it has enabled the protection of the kernel from moulds and insects during growth stage. In case this hull is ruptured, this protection is not provided anymore, the shell is stained and the kernel can be infected by moulds. The cause of shell staining might be due to the high content of phenolic compounds in the hull (Labavitch *et al.*, 1982). Ferguson *et al.* (1995) reported that shell staining generally increases with increased temperatures and increased holding times. There are two types of hull ruptures, resulting either in early split or cracked pistachios. In the first case, in the orchard, some nuts have the hull attached to the shell so that the hulls split with the shell (Doster *et al.*, 1993). In such cases, the hull splits along the shell suture, resulting in pistachio shells stained along the shell suture. These nuts are called early splits and may be contaminated by aflatoxin (Sommer *et al.*, 1986). However, all early split pistachios do not form at the same period. Doster and Michailides (1995) reported that even though the splitting of shell and hull for early splits tended to precede the shell splitting of normal nuts, a number of early splits formed after many normal nuts had split their shells. Even normal pistachio nuts matured unevenly over a period of several weeks during which shells split. Depending on the orchard, 15 to 48% of the early splits had formed more than 4 weeks before harvest and 10 to 30% formed within 2 weeks before harvest. Not all early splits are infected by *Aspergillus* fungi, and the incidence depends on the date of splitting (Doster and Michailides, 1995). It results in even a higher variability of AFB1 contamination levels among early splits: some are contaminated while others are not. This is due to the fact that among all *Aspergillus* species, only *A. flavus* and *A. parasiticus* toxigenic strains have the ability to produce the toxin, and this, only under specific conditions. Furthermore, contamination levels vary between contaminated early split pistachios. The same kind of contamination variability is observed for cracked pistachios, and for the same reasons. Cracking of hulls is another type of hull rupture. This kind of hull split takes place in less than 15 days of harvest and is characterized by ragged brown edges and much wider splits than early splits and random orientation of split on hull (Pearson *et al.*, 1994). Indeed, in cracked fruits, the location of the rupture of the hull does not correspond to the split in the shell. Early split and cracked pistachios can be either contaminated by AFB1 or not, as well as exhibit internal brown spotting or visual mold growth or not, depending on individuals. Brown spotting or visual mould growth might possibly be indicators of AFB1 contamination; however it is not always the case. Moreover,

sometimes early split or cracked pistachios having apparently sound kernels are contaminated. And on the contrary, pistachios having suspect kernels and/or split hulls might not be contaminated. For these reasons, as mentioned earlier, for modeling purposes, we only considered early split pistachios having sound kernels and cracked pistachios having sound kernels mentioned as early split sound kernel category and cracked sound kernel category, respectively. Steiner *et al.* (1992) observed that some brown or brown spotted pistachio kernel sections in pistachio nuts cut lengthwise with a knife, were highly contaminated with aflatoxin. These spots can be either typical kernel necrosis symptoms or indication of fungal growth. As regards kernel necrosis symptoms, they are caused by large bugs' feeding named hemipteras. According to Michailides *et al.* (1995), kernel necrosis symptoms differ from stigmatomycosis, which is characterized by the wet, smelly, rancid, slimy appearance of the kernel. Typical kernel necrosis symptoms caused by hemipteras are dry, punchy, areas in the kernel, spongy at times, usually appearing in the area close to the stem and or along the split line of the shell (Danisti *et al.*, 2001). The most critical period for potential kernel damage occurs in midseason, when fruit load is set, shell hardening is not complete and large bugs' mouthparts can easily penetrate hardening shells. The damaged nut remains in the cluster and the feeding punctures result either to epicarp lesion staining the outer shell or to necrotic spots on the kernels contaminated by fungi (Daane *et al.*, 2005a). The subsequent feeding by large bugs presents potential "hidden" damage, because kernel necrosis may occur without the external sign (hull lesion) of insect feeding. Late-season feeding can also lead to kernel necrosis when the insect's mouthparts penetrate the shell near the "Achilles heel" of the pistachio, the region near the peduncle (nut's stem) (Michailides *et al.*, 1988). The contamination pathway of pistachios by *A. flavus* or *parasiticus* following bugs' feeding is probably the same as by another fungus, *Botryosphaeria dothidea* (Bd), a panicle blight of pistachios. Daane *et al.* (2005b) reported that for Bd, the most probable contribution of bugs is providing germination sites for the fungus: by puncturing the epicarp with their mouthparts, the bugs allow spores that are already present to germinate. Hadavi (2005) concluded that in-orchard contaminations probably obey the same rules as inoculation tests, which are, i.e. the fungus gains entry from any kind of splits in hull and grow between hull and shell space until reaching to nut stem and after penetrating via numerous microscopic passages here, the fungus reaches the kernel in the placental region. The fungus possibly follows the least physical restriction with adequate moisture path, which directs it to intra-cotyledonary space, the observed brown colour or spots here, probably may act as an indicator of physical growth and/or restriction pattern of fungus (Hadavi, 2005). In our work, we put pistachios having brown or brown spotted pistachio kernel sections under the mouldy category.

## 2.2. Global Steps of the Method

The modeling method developed in this work used the Monte Carlo technique, and comprised many steps as described hereafter. The cumulative distribution functions of contamination levels in individual pistachios corresponding to each category (early split sound kernel; cracked sound kernel; mouldy) were shown to be linear with a positive slope in graphical representations with lognormal distribution probability scales. The lognormal distributions were then used to calculate 30 kg samples contamination levels for a given number of contaminated

individuals per sample. Differences between good and bad lots were taken into account with lots split into 3 quality lot classes. The following calculations were achieved for each quality lot class separately, knowing that the various pistachio category incidences vary between quality lot classes. First, the probability of having a specific number of contaminated individuals within each specific category part of a 30 kg sample was determined thanks to a binomial distribution, taking into account the contamination incidence. Then, a probability distribution was used to combine probabilities and contamination level distributions formerly calculated. As a result, 30 kg samples contamination distributions, which take all the possible number of contaminated individuals into account, were obtained. Finally, these contamination distributions for each category were added up, thereby giving the distribution of 30 kg samples contamination levels for each quality lot class. Following this, the three obtained distributions (one for each quality lot class) were combined thanks to binomial distributions: the two lowest quality lots classes were first combined, and then the result was combined to the highest quality lot class.

### 2.3. Contamination Incidence and Levels

Contamination incidence and levels in individual pistachios were calculated from results reported in a publication from Hadavi (2005), in which all aflatoxin contaminations were due to *A. flavus*, thus resulting in AFB1 contamination. In the study we refer to, pistachios were collected from six commercial orchards in Iran and aggregated into samples, based upon hull appearance. It is reported that, by this sampling procedure, the heterogeneous distribution of contaminated nuts was well covered, as practiced earlier in literature (Hadavi, 2005). Sound, early split, cracked (growth split) pistachio samples for each orchard were separated according to fluorescence or non-fluorescence, as well as kernel aspect. Indeed, the bright greenish yellow colour under an ultraviolet light, referred to as the 'BGY' fluorescence (BGYF) is partially linked to aflatoxin contamination (Hadavi, 2005). The published data taken into account for our simulation, but with slight modification, were sample contamination levels for early split and cracked pistachio samples having sound kernels, as well as pistachios having brown spotted or mouldy kernels, or with the presence of fungal colony. More precisely, in our work, pistachios were categorized according to visual defects into three categories, namely early split sound kernel, cracked sound kernel and mouldy pistachios. As the sample size was small enough (from 1.1 g to 168 g of kernels), we considered that a maximum of one kernel per sample was contaminated, taking into account a kernel weight of 0.52 g. Indeed, aflatoxin incidence is a rare event in pistachios, even in mouldy kernels, due to the fact that *Aspergillus* is not the sole mould capable of infesting the nut. In addition, when toxigenic *Aspergillus* is present, toxin is only synthesized in specific conditions. Then, the calculation of the incidence of contaminated pistachios per category and individual contamination levels was achieved. Individual contamination level distributions were fitted to theoretical distributions for each pistachio category. We made the hypothesis, for the contamination incidence calculation, that a maximum of one kernel per sample was contaminated. In order to further validate this assumption, we compared the contamination incidence of early split pistachios (having sound or brown spotted kernels) calculated from Hadavi (2005) data to the incidence level calculated from other publications. This latest calculation was made by multiplying the incidence level of 2% of pistachios with *A. flavus* among early split pistachios

reported by [Bonjar \(2004\)](#) for the 2003 crop year by the proportion of toxigenic fungi among *A. flavus* isolates from pistachios. Indeed, [Rahimi et al. \(2008\)](#) reported that among 150 isolates of *A. flavus* tested for aflatoxin production, 46 were identified as aflatoxin producing isolates. Calculated individual contamination levels were compared to data from a publication from [Mahoney and Rodriguez \(1996\)](#). In their study, they used mature pistachios with undamaged and nonadhering hulls, collected from an orchard for in vitro inoculations. Their protocol is in the following paragraph:

The hulls were aseptically sliced along the shell suture to simulate early-split pistachios on 100 nuts, and pistachios with unopened shells were discarded. The hulls and shells were aseptically removed from other 200 nuts. One-half of all kernels were injured by using a scribe to make a puncture wound approximately 1 mm wide by 2 mm deep in each kernel. Pistachios were placed in petri dishes (five nuts per plate). Each nut was inoculated on the kernel (or the wound in the kernel) with approximately 200 spores of *A. flavus* aflatoxigenic strain. The petri plates were incubated for 10 days at 25°C.

From these published data, we transformed aflatoxin levels in individual pistachios given in µg/kernel in order to obtain concentration levels in µg/kg. Then, data for contaminated hull sliced pistachios (intact or wounded kernels) were fitted to a theoretical distribution. Mean and standard deviation obtained were compared to our simulation results calculated for the early split sound kernel category. We considered that the theoretical distribution of contaminated hulled pistachios would be the same as for hull sliced pistachios. We simulated the distribution for hulled intact kernel pistachios and hulled wounded kernel pistachios, using @Risk (version 6, Palissade Corp.). Finally, we combined the two distributions, using a binomial distribution. Mean and standard deviation of the resulting distribution were compared to our simulation results for mouldy category pistachios. Another validation was made by comparing the incidence of highly toxigenic *A. flavus* strains in early split pistachios (resulting in a concentration level > 200 µg/kg in a pistachio) calculated from the contamination incidence calculation under study for Iranian pistachios to the same kind of data from USA. Indeed, [Pearson \(1996\)](#) reported for the USA, from a publication from [Sommer et al. \(1986\)](#), that the incidence of aflatoxin contamination greater than 200 µg/kg in individual early split nuts was approximately 1 in 270. Knowing the incidence of early split pistachios contaminated by *A. flavus* (either toxigenic or not) for both countries: 2% in Iran ([Bonjar, 2004](#)), 0.7% in USA ([Doster and Michailides, 1994](#)), we deduced the proportion of early split pistachios with *A. flavus* that have a highly toxigenic strain of the fungus. We assume that the incidence of a highly toxigenic *A. flavus* in individual early split pistachios contaminated by *A. flavus* is supposed to be the same in both countries (Iran and USA). If so, it validates the strategy used in this work for contamination incidence and levels obtainment.

#### 2.4. Monte Carlo Technique Using @Risk

The @Risk software (version 6, Palissade Corp.) is a Monte Carlo software for risk analysis and risk assessment using Monte Carlo random simulations. It is used to determine how often specified events may occur thanks to the generation of Probability Density Functions (PDFs). This probability risk analysis approach is opposed to the deterministic risk analysis calculation. Indeed, when achieving a deterministic risk evaluation, values are assigned for discrete scenarios to see

what the outcome might be in each. On the contrary, in a probability risk evaluation, values are given to all the possible scenarios, taking into account their probability of occurrence. It is used to estimate the effect of variability on the outputs of models which use a variety of input parameters. Monte Carlo simulation is the replication of a procedure with input data drawn randomly from a parametric distribution. However, Monte Carlo simulation can be used with two different sampling types under @Risk: the Monte Carlo (MC) and the Latin Hypercube (LH) sampling types. The LH sampling is a particular MC sampling: more precisely it is a constrained MC (i.e. random) sampling scheme, involving a stratification of the input distribution. The constraint refers to the way each variable is sampled: the statistical cumulative distribution is split in  $n$  equally probable intervals on the cumulative probability scale, and then a random value is selected within each interval. The number of  $n$  equally probable interval is equal to the number of iterations of the Monte Carlo simulation. A simulation with 500 iterations, per example, would split the probability into 500 segments, each representing 0.2% of the total distribution. For the first segment, a number would be chosen between 0.0% and 0.2%. For the second segment, a number would be chosen between 0.2% and 0.4%. This number would be used to calculate the actual variable value based upon its distribution. Once each variable has been sampled using this method, a random grouping of variables is selected for each Monte Carlo calculation. Independent uniform selection is done on each of the variable's generated values. Each value must only be used once. LH sampling would create a more representative sampling of the distribution, ensuring that tail samples of the input distributions are drawn (e.g. for 1000 iterations, exactly one value above the P99.9 would be drawn, whereas for MC sampling either none, one or several samples may be drawn). So, LH sampling can be used to force the sampling of tails of distributions, along with a great number of iterations. This is why, in this study, thanks to the @Risk 5.7 software (Palisade Corp.), PDFs were modeled, using Monte Carlo simulations under the LH sampling type. The number of iterations was 100,000 to attain a very high stability level.

## 2.5. Modeling of Pistachio Categories and Contamination

Simulated lots were split into three different quality levels for the mean contamination level, numbered by ascending contamination level: 1<sup>st</sup> class, 2<sup>nd</sup> class, 3<sup>rd</sup> class. We considered a constant incidence level of pistachio categories within lots of the same quality levels. The incidence of pistachio categories used for pistachios was deduced from a document from the UNECE (United Nations Economic Commission for Europe), defining the maximum tolerances for « extra pistachios » (UNECE, 2001): 2% of early split pistachios and 1% of cracked pistachios. So, for 2<sup>nd</sup> and 3<sup>rd</sup> class lots, the incidence levels of early split sound kernel and cracked sound kernel pistachios were set at 2% and 1% respectively. However, for 1<sup>st</sup> class lots, these categories incidences were divided by 2. As regards the incidence of the mouldy pistachio category, we kept it as an adjustment variable. The final calculation of contamination incidence was achieved thanks to the Bayes' theorem in Equation (1):

$$P(\text{contaminated} \cap \text{category}) = P(\text{contaminated} / \text{category}) \times P(\text{category}) \quad (1)$$

with  $P(\text{contaminated})$ , the probability that the individual is contaminated and  $P(\text{category})$ , the probability that the individual is in a given category.

Calculations were achieved for each pistachio category separately, at first. The contamination distribution for 30 kg samples for a given number of contaminated individuals per sample is given in Equation (2):

$$C_s = \frac{C_i}{N_i} \times N_{ic} \quad (2)$$

With  $C_s$ , 30 kg samples concentration distribution;  $C_i$ , individual pistachios concentration distribution;  $N_i$ , the number of individual inshell pistachios in a 30 kg sample;  $N_{ic}$ , the number of contaminated individual inshell pistachios in a 30 kg sample. The probability of a given number of contaminated individuals (0, 1, 2, 3, etc.) was calculated thanks to a binomial distribution, knowing the final calculation result of the contamination incidence. These probabilities were then grouped together. A conditional function was used to match these probabilities with concentration levels distributions in 30 kg samples. The result was a PDF for the contamination level in 30 kg samples, considering only the contamination stemming from one pistachio category. In a second step, PDFs obtained for each pistachio category were added up in order to obtain the PDFs of final concentration in 30 kg samples for each of the 3 classes. The three PDFs obtained for the quality lot classes were combined thanks to binomial distributions. More precisely, the two lowest quality lots classes (2<sup>nd</sup> and 3<sup>rd</sup> class) were first combined, and then the result was combined to the highest quality lot class (1<sup>st</sup> class). However, various probability values to use for the binomial distributions were tested. The values chosen were the ones that gave a final PDF for contamination levels in 30 kg samples as close as possible to the observed values in Iran as further detailed in 2.6.

## 2.6. Simulation Adjustment

Our simulation final parameters were the binomial probabilities to put the three quality lot classes together. These parameters were chosen in such a way that the resulting output final PDF for contamination levels in 30 kg samples was adjusted to a contamination distribution observed in Iran, reported by [Cheraghali et al. \(2007\)](#). Their distribution was obtained for pistachio nuts produced in Iran during March 2002–February 2003 and analyzed for AFB1. Lots sampled were pistachio nut consignments, intended for export to EU, that are usually about 25 tons. Their sampling plan was the following: taking one 30 kg sample (100 incremental samples, each 300 g), mixing and division to three 10-kg sub-samples. They collected 3,356 pistachio nut samples of 30 kg, and after dividing samples to 10 kg sub-samples, performed 10,068 AFB1 analyses. However, the mean of the contamination level in sub-samples was used in their study, which is the same as if they had analyzed 30 kg samples directly.

## 3. RESULTS AND INTERPRETATION

### 3.1. Contamination Incidence and Levels

Contamination levels in individual pistachios were calculated from contamination levels in small samples reported by [Hadavi \(2005\)](#) for contaminated samples only. Results are given in Table 1 and were obtained by multiplying contamination levels in the samples by the corresponding number of pistachios in the samples (deduced from total kernel weight) for each pistachio category. Furthermore, the calculation of the incidence of contaminated pistachios per



category was achieved:  $P$  (contaminated / category)), it says the probability that an individual pistachio was contaminated, knowing that it was in a specific category. Considering that a maximum of one kernel per small sample was contaminated, in agreement with section 2.3, the result for the probability of contamination within each category, was equal to  $7.53425 \times 10^{-3}$ ,  $6.709265 \times 10^{-2}$ , and  $6.14035 \times 10^{-3}$ , for early split sound kernel, mouldy, and growth split sound kernel categories, respectively. Goodness of fit tests were achieved for calculated individual contamination levels (reported in Table 1) for each pistachio category, using @Risk. Test statistics for the Kolmogorov-Smirnov (K-S) test for lognormal distribution, triggered by the software were compared to critical values for this test at a 5% risk level. As each test statistic value is lower than its corresponding critical values, a lognormal distribution cannot be rejected as the true parent distribution at a 5% risk level. For a visual assessment, cumulative distribution functions (CDF) for contamination levels, were plotted using the R software in Web Figure 1, 2, 3 for early split sound kernel, cracked sound kernel and mouldy pistachio categories, respectively, in graphs with a standard normal probability scale and a log axis for the data. Least square regression lines were obtained for each graph and show a low dispersion level. As, with such a graphical representation, any given lognormal CDF plots as a straight line with positive slope, we further confirm that the distributions under study fit lognormal distributions. Mean contamination level was at  $5542 \mu\text{g}/\text{kg} \pm 10525 \text{ SD}$ ;  $2527 \mu\text{g}/\text{kg} \pm 3412 \text{ SD}$ ; and  $287646 \mu\text{g}/\text{kg} \pm 706790$  for contaminated early split sound kernel, cracked sound kernel, and mouldy pistachios, respectively. So, mean individual contamination level is higher in early split sound kernel than in cracked sound kernel pistachio category, the highest mean contamination being in mouldy pistachio category.

Indeed, [Doster and Michailides \(1994\)](#) reported that most early split nuts had the hull split before any pistachio fruits had cracked hull, which may explain why cracked fruits have less kernel mould than early splits. The fact that early split and cracked pistachio categories (having apparently sound kernels) are contaminated can be explained by the fact that the mould is not present anymore, but has synthesized aflatoxins while it has been growing. Considering a maximum of one contaminated kernel per sample in data from Hadavi's publication, we calculated the contamination incidence of early split pistachios (having sound or brown spotted kernels), as being 23 in 1672, leading to a value of 1.37%. This value was compared to another calculation result of the same parameter derived from [Bonjar \(2004\)](#) and [Rahimi et al. \(2008\)](#):  $0.30 \times 2\% = 0.60\%$ . As 1.37% is in the same order of magnitude as 0.60%, we can deduce that contamination incidence values, as calculated for our simulation must be very close to what could have been observed if individual pistachios had been analyzed for aflatoxin. Indeed, aflatoxin analyses are very costly and thus seldom achieved on individual pistachios. And even though individual pistachios were analyzed in few literature data, these analyses were only achieved on a limited number of individuals. For this reason, we consider contamination data from small samples for our simulation. As the calculated incidence from small samples reported above fit well with calculated incidence derived from fungal incidence and toxicity expectations, we can deduce that our contamination incidence calculation thanks to small samples data is validated. Calculated individual contamination levels for our simulation were compared to in-vitro data from a publication from [Mahoney and Rodriguez \(1996\)](#). Their contamination data for hull sliced

pistachios, having the same aspect as early splits, were fitted to a lognormal distribution, for contaminated individuals only, using @Risk. The test statistic value for the Kolmogorov-Smirnov (K-S) test was equal to 0.0998. It is lower than the critical value (0.1831) for this test at a 5% risk level. So, a lognormal distribution cannot be rejected as the true parent distribution at a 5% risk level. Mean level obtained was at 4565 µg/kg, with a standard deviation (SD) equal to 6092. These values are very close to the corresponding parameters obtained with our simulation for early split sound kernel category. Furthermore, their data for contaminated hulled pistachios were tested too. We considered that both hulled intact kernel and hulled wounded kernel contamination levels would fit a lognormal distribution. So, we simulated the distributions for hulled intact kernel pistachios (mean = 46519 µg/kg; SD = 164942) and hulled wounded kernel pistachios (mean = 557692 µg/kg; SD = 430153), using @Risk. Then, we combined the two distributions, using a binomial distribution, with a probability set at 0.5. The resulting distribution (mean = 299502 µg/kg; SD = 406013) is very similar to the one obtained with our simulation for mouldy pistachio category. Thanks to all these results, we can validate the process used for our simulation, for contamination level distribution obtainment. From Hadavi's data, the incidence of aflatoxin contamination greater than 200 µg/kg in individual early split nuts was 15 in 1672, corresponding to a result of 1 in 111. Knowing that the incidence of early split pistachios contaminated by *A. flavus* (either toxigenic or not) in Iran was 2% (Bonjar, 2004), we calculated that 45% of early split pistachios contaminated by *A. flavus* were contaminated by a highly aflatoxigenic strain. The same calculation was made for pistachios from USA, as described in paragraph 2.3., and the corresponding final result was 53% for the proportion of early split pistachios contaminated by *A. flavus* that are contaminated by a highly aflatoxigenic strain. As both figures for Iran and USA are rather similar, we can validate the strategy used in this work for contamination incidence and levels obtainment.

### 3.2. Contamination Levels Distribution in Samples

As reported in paragraph 2.5, for 1<sup>st</sup> quality lot class, the incidence levels of early split sound kernel and cracked sound kernel pistachios were set at 1% and 0.5%, respectively. For 2<sup>nd</sup> and 3<sup>rd</sup> class lots, the incidence levels of early split sound kernel and cracked sound kernel pistachios were set at 2% and 1%, respectively. In order to cover the whole range of AFB1 levels observed in 30 kg samples in the publication used for simulation adjustment (reported in paragraph 2.6.), incidence levels of mouldy pistachios were set at 0%, 0.01%, and 0.25% for 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> quality lot classes. The corresponding calculation result of final contamination incidence, achieved with Equation (1) and probability of contamination data, is shown in Table 2. Then, calculations were achieved as described in paragraph 2.5. Thanks to these data, PDFs were simulated using @Risk, for each quality lot class, as described below. The contamination PDF in 30 kg samples from 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> quality lot class are given in Figure 1, 2 and 3, respectively. In order to enable the comparison with Cheraghali's data, the proportion of samples in various intervals is given in Table 3. When comparing the 3 quality lot classes' distributions given in Table 3, it is noticeable that, for the 3 highest contamination intervals, 3<sup>rd</sup> quality lot class samples might have the greatest impact, if all 3 quality lot classes were put together. Indeed, the 3 quality lot classes had to be mixed up to fit simulation data to observed results (Cheraghali's data), but prior to this, it

was important to ascertain that the whole range of observed concentrations is well covered. In the same way, for the lowest contamination interval, both 1<sup>st</sup> and 2<sup>nd</sup> quality lot classes have probably the highest impact, whereas for 2.01 – 5 µg/kg and 5.01 – 20 µg/kg intervals, both 2<sup>nd</sup> and 3<sup>rd</sup> quality lots have the biggest weight. These observations are further examined in Web Figure 4.

From this figure, we observed that the range of observed concentrations (in Cheraghali's data) is well covered by the 3 simulated quality lot classes. However, simulation adjustment reported in paragraph 2.6 had to be achieved, to combine the 3 quality lot classes together.

### 3.3. Final Simulation Adjustment and Final Contamination Levels in Samples

A binomial distribution was used to combine the 2<sup>nd</sup> and 3<sup>rd</sup> lot classes together with a probability at 0.6 for the 2<sup>nd</sup> lot class. This combination was then mixed with the 1<sup>st</sup> lot class, this latter class having a probability set at 0.8. Simulated and observed (from Cheraghali's data) PDFs for final concentrations in 30 kg samples in export packages are compared in Web Figure 5. For 30 kg samples above 500 µg/kg, the observed percentage was 0.07% and the simulated percentage at 0.1%. The simulation was validated, as the probabilities were very similar in both simulated and observed distributions. Moreover, obtained mean and SD for the simulation were equal to 5.2 and 45.9 µg/kg, respectively, whereas the corresponding values from observed PDF were 5.9 and 41.7 µg/kg, respectively.

## 4. DISCUSSION

Due to the low contamination incidence in individual pistachios, AFB1 concentration levels differ greatly between 30 kg samples. Our strategy in this modeling work is to split lots between quality classes as regards concentration levels. These categories are chosen as they are highly susceptible of being contaminated by AFB1. We postulate that using analytical data for small samples of each pistachio category and then deriving the concentration levels in 30 kg samples, as achieved in our study, is more reliable than using directly 10 kg or 30 kg samples analytical data, as usually performed in Whitaker's method. As the contaminant is heterogeneously distributed, it appears that small samples of pistachios having the same defects must have a more homogeneous contamination distribution than big samples of any pistachio type (not split into categories). Furthermore our simulation method uses the Monte Carlo technique, thereby enabling random simulations, as well as the combination of various distributions. When our results are compared to contamination levels observed in Iran, both distributions are very similar. A sensitivity analysis must be performed with @Risk, to show the sensitivity of the output contamination distribution to its input variables. The goal is to identify and rank important sources of variability. The only parameters that are set in the simulation in order to fit the final observed distribution and that are used as adjustment variables are the binomial probability levels. Indeed, these binomial probabilities are used to combine the 3 quality lot classes' distributions together. All the other parameters, used in our model, are obtained or derived from analytical data or regulatory levels. For this reason, it is necessary to assess whether the binomial probability levels are important sources of variability in the model or not. Sensitivity analysis results show that the regression coefficient between the output distribution and these two input variables have a global value of 13%. So, the two input variables are low sensitivity variables, meaning that even large

changes to the distribution of these variables may have a minimal impact on the final result. In other words, the binomial probability levels used do not have a high influence on the output contamination distribution. Then, a sensitivity analysis targeted on the variation of these two input variables was achieved. In order to better comprehend and quantify the impact of the binomial probability levels chosen, we set them at different values and observed the effect on the output contamination distribution. The binomial probability levels for each variable were set at their initial value  $\pm 0.1$ , their initial value being the value used for final simulation adjustment (paragraph 3.3). So, for the first input variable under study, the probability value levels tested for the 2<sup>nd</sup> lot class were 0.5, 0.6 or 0.7. As regards the second input variable, the probability value for the 1<sup>st</sup> lot class was set at 0.7, 0.8 or 0.9. All the output distributions for final contamination levels from our model, resulting from each of the possible combinations of the two variables under study were simulated using @Risk. The percentage of samples for various contamination levels was obtained. Then, the minimum and maximum percentage values for each contamination interval level were compared to the percentage values for the model previously validated (paragraph 3.3). Results are plotted in Web Figure 6, with minimum and maximum values represented as lines on the bars to show some aspects of variability on the developed model. This sensitivity analysis confirms that when the two binomial probability levels vary, the impact on the output distribution is very low. Thus, the model developed in our study is robust. The impact of this work might be interesting from a prevention point of view, as shown hereafter. The model is based on contamination levels in specific pistachio categories, and the proportion of these categories within a lot is function of the mean contamination level of the lot. So, estimating the proportion of pistachio categories within each lot gives an estimation of lot mean contamination level.

This simulation uses AFB1 contamination levels from early split pistachios and cracked pistachios having sound kernels, as well as from pistachios having brown-spotted kernel sections due to kernel necrosis or mould infection. All these pistachios are dubious nuts, meaning that they are highly susceptible of being contaminated. Observed contamination levels distribution in samples taken from lots from Iran, come from the presence of these nuts. Indeed, contamination levels per contaminated pistachios within a sample are cumulated and must be divided by the total sample weight to obtain sample concentration level.

## 5. CONCLUSION

Contamination incidence and levels in individual pistachios used in our simulation were validated thanks to calculations made from other literature data. They were then combined together to simulate contamination PDFs in 30 kg samples of three different classes of pistachio lots. The final result was the contamination distribution in 30 kg samples, adjusted thanks to contamination data from Iran. So, as the simulation truly reflects observed contamination levels distribution in samples from Iran, it validates our understanding of the points that bring contamination variability. By this way, this work is going to enable to predict sample distribution better and thus to validate sampling plans more accurately.

## 6. ACKNOWLEDGEMENTS

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**Table-1.** Contamination levels in individual pistachios from each of the three pistachio categories considered

contamination levels (µg/kg)				
early split sound kernel		mouldy		growth split sound kernel
7	452	1.5	5333.44	7.88
14	971	2.68	5353.29	109.71
22.88	2662	24.16	7370.58	203.55
58.08	3013	27.8	8608.48	289.56
72	3270	147.3	9256.8	2662.58
109	5897	512.82	9559.76	5897.25
175	8523	825.12	140828.8	8520.2
203	26349	877.37	1418847	
289	29363	942.92	1546770	
318.08	34185	5237.82	2592381	
445				

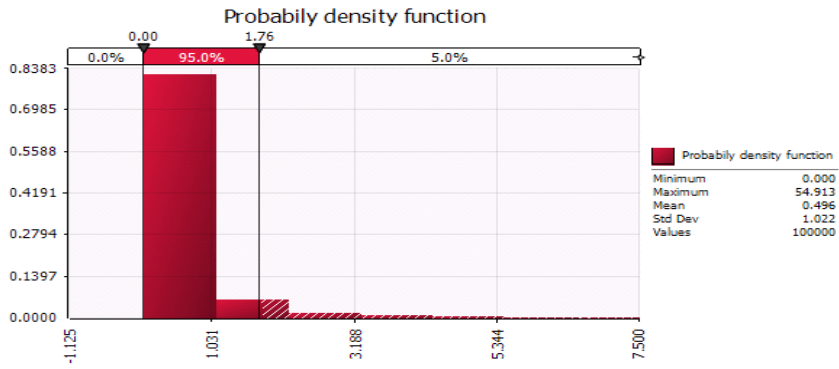
**Table-2.** Final calculation of contamination incidence:  $P(\text{contaminated} \cap \text{category})$

final contamination incidence			
pistachio category	1 <sup>st</sup> class	2 <sup>nd</sup> class	3 <sup>rd</sup> class
early split	$7.5456 \times 10^{-5}$	$1.50912 \times 10^{-4}$	$1.50912 \times 10^{-4}$
cracks	$3.05666 \times 10^{-5}$	$6.11332 \times 10^{-5}$	$6.11332 \times 10^{-5}$
mouldy	0	$8 \times 10^{-6}$	$1.69074 \times 10^{-4}$

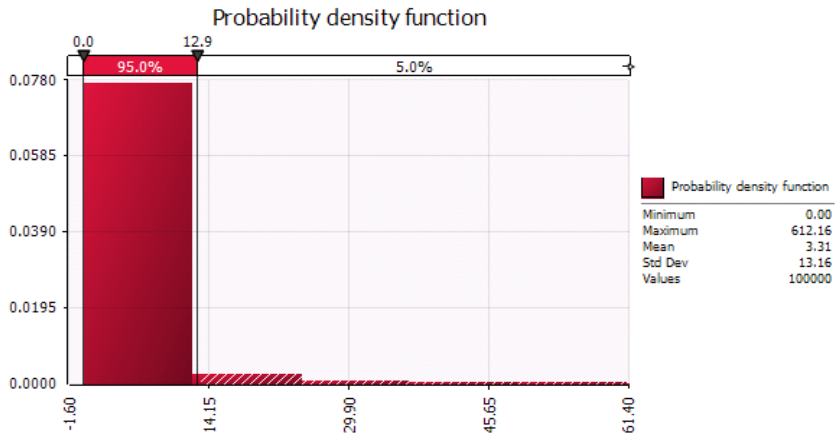
**Table-3.** AFB1 contamination level distribution for 30 kg samples for each quality lot class

AFB1 contamination level interval (µg/kg)	proportion of samples		
	in 1 <sup>st</sup> quality lot class	in 2 <sup>nd</sup> quality lot class	in 3 <sup>rd</sup> quality lot class
0 - 2	96.1%	74.5%	5%
2.01 - 5	3.2%	12.8%	12.8%
5.01 - 20	0.7%	9.6%	36%
20.01 - 50	0%	2.1%	22.7%
50.01 - 500	0%	0.8%	22.1%
> 500	0%	0%	1.1%

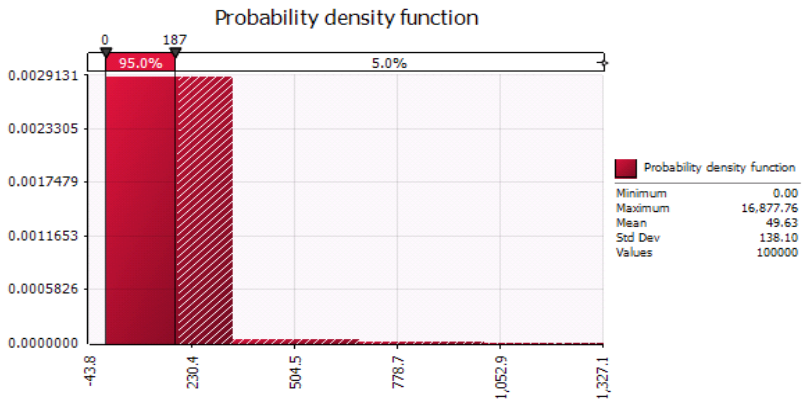
**Figure-1.** AFB1 contamination ( $\mu\text{g}/\text{kg}$ ) PDF for 30 kg samples from 1<sup>st</sup> quality lot class



**Figure-2.** AFB1 contamination ( $\mu\text{g}/\text{kg}$ ) PDF for 30 kg samples from 2<sup>nd</sup> quality lot class



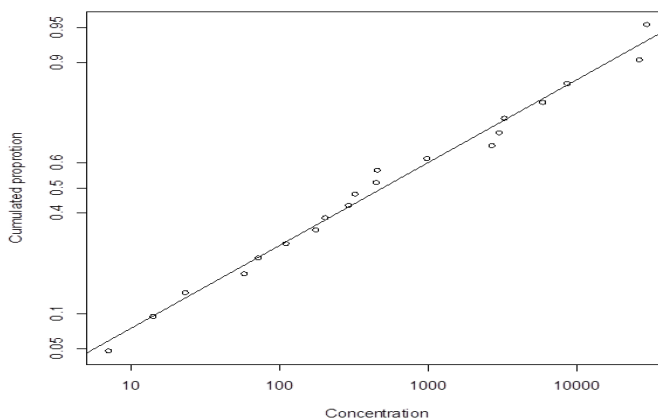
**Figure-3.** AFB1 contamination ( $\mu\text{g}/\text{kg}$ ) PDF for 30 kg samples from 3<sup>rd</sup> quality lot class



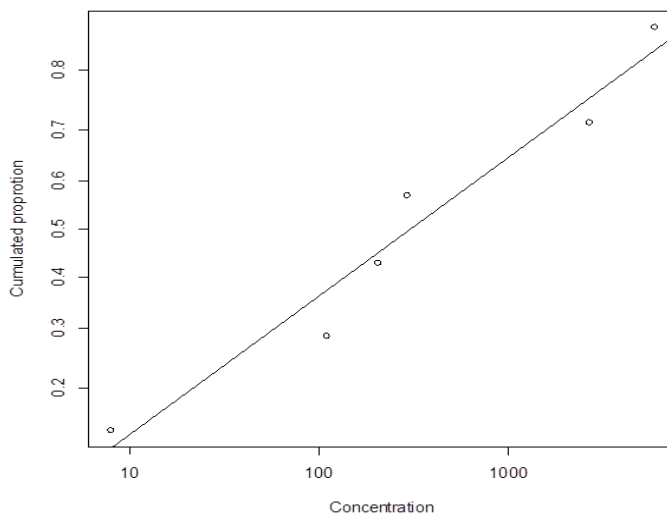


## APPENDIX

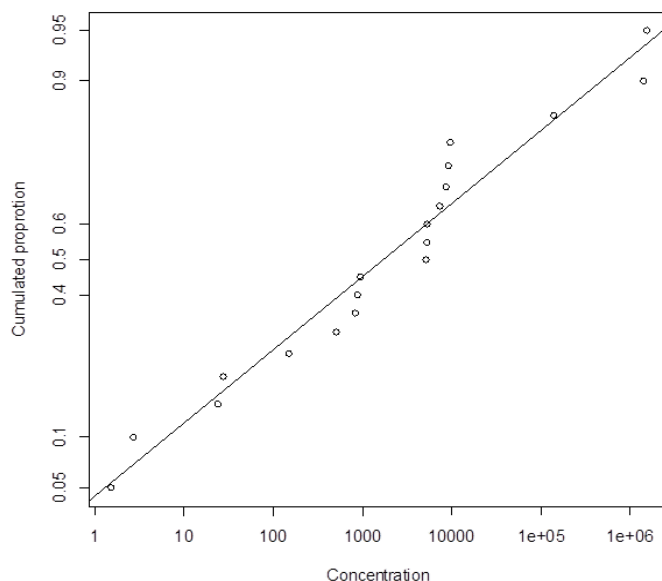
**Web Figure-1.** Cumulative distribution function for the early split sound kernel pistachio category with aflatoxin concentration in  $\mu\text{g}/\text{kg}$



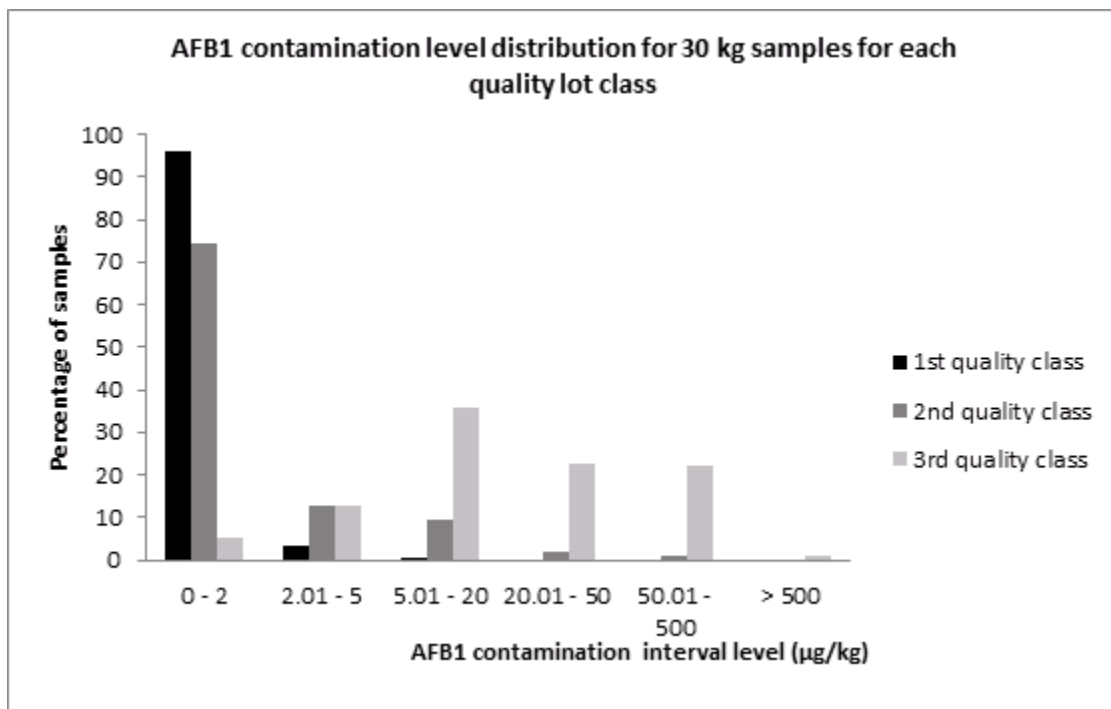
**Web Figure-2.** Cumulative distribution function for the cracked sound kernel pistachio category with aflatoxin concentration in  $\mu\text{g}/\text{kg}$



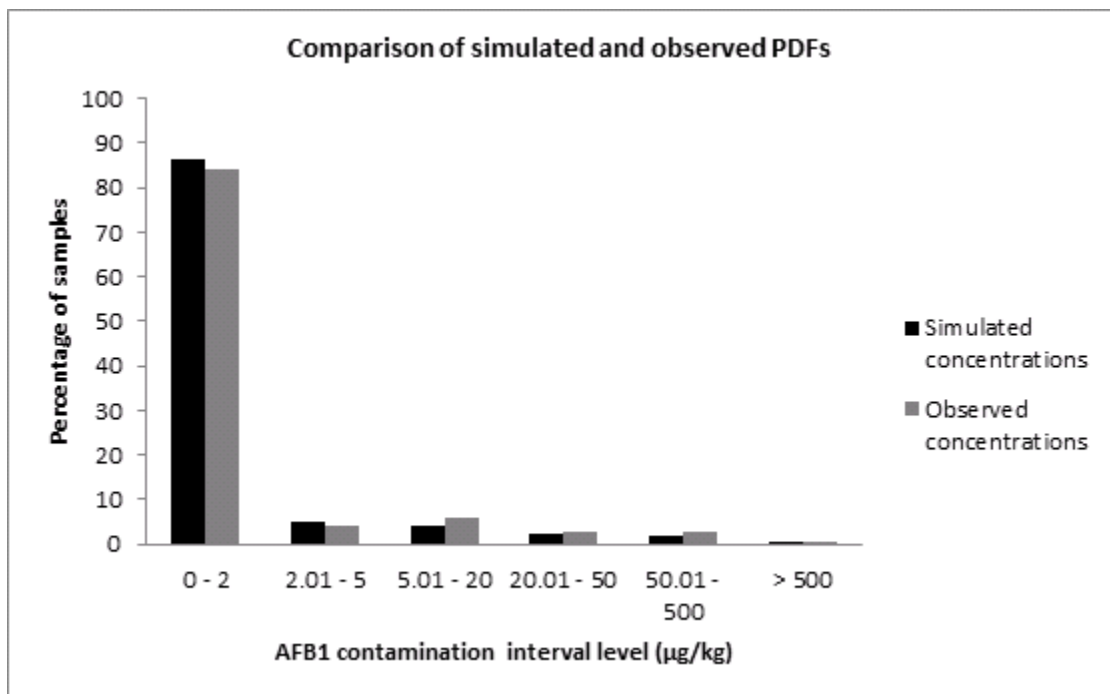
**Web Figure-3.** Cumulative distribution function for the mouldy pistachio category with aflatoxin concentration in  $\mu\text{g}/\text{kg}$



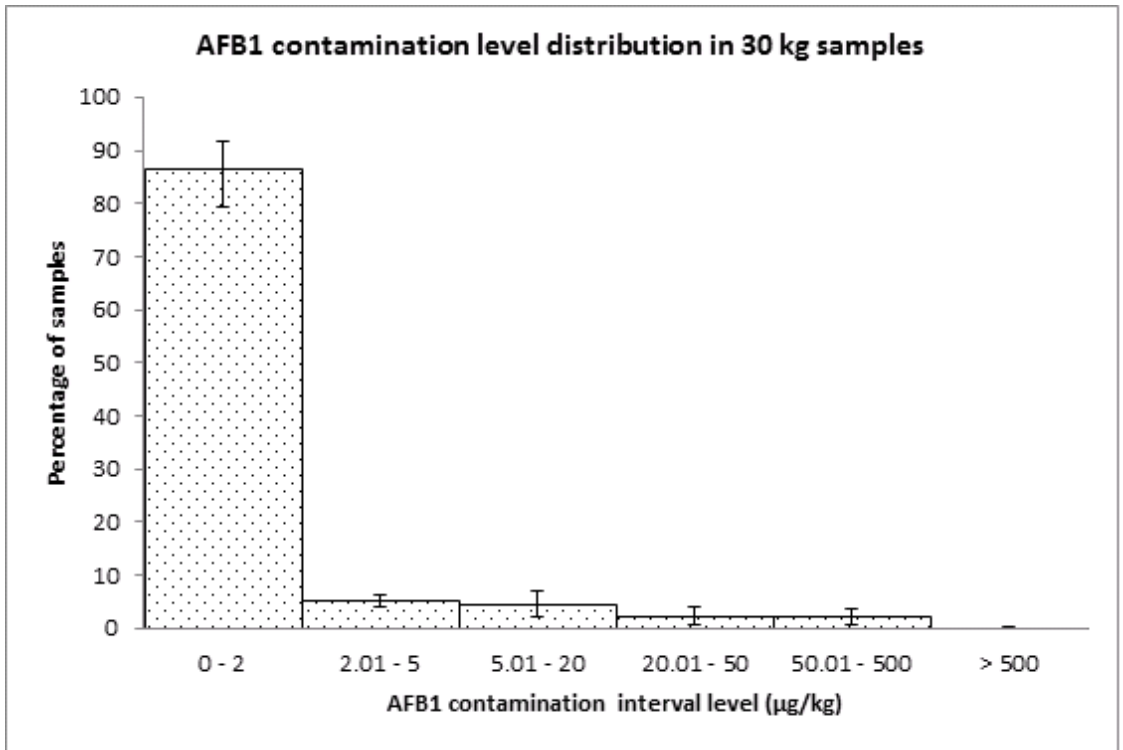
Web Figure-4. Visualization of the 3 quality lot classes' impact on contamination level



Web Figure-5. Visual validation of final simulation results distribution



**Web Figure-6.** Impact of various probability levels tested for the two input binomial variables on the output distribution



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