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Changes in Heterogeneity of Aflatoxin Distribution During Processing Coconut Oil from Copra

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ABSTRACT

Background and Objective: Aflatoxin distribution in nuts and seeds is highly uneven, which makes test results unreliable and can cause errors of up to 90%. This variability depends on kernel size and surface area. Copra (smoked coconut kernels) provides a large surface for fungal growth and its oil is an important dietary source in coconut-growing countries. The study aimed to determine the most suitable point of sampling for accurate aflatoxin quantification.

Materials and Methods: Aflatoxin B1 levels were measured by quantified in contaminated copra kernels, steam-cooked copra pieces, expelled coconut oil and copra cake collected from 44 oil mills in Sri Lanka over one year. A total of 414 samples were analyzed.

Results: The coefficient of variation (CV) for aflatoxin B1 decreased progressively across the processing stages: 2.6 in moldy copra kernels, 1.4 in copra pieces, 1.0 in oil and 1.1 in copra cake. This shows that size reduction and mixing improve sampling accuracy. Some visibly moldy kernels tested negative for aflatoxins.

Conclusion: Accurate aflatoxin detection depends on when, where and how samples are collected. Sampling coconut oil provides a more reliable measure of aflatoxin hazards compared to testing raw copra kernels.

INTRODUCTION

Mycotoxin contamination of agricultural products is a major food safety hazard associated with the agri-food chain. Aflatoxin production and accumulation in the agricultural raw materials occur during crop growth and maturation as a pre-harvest phenomenon, or during storage due to inadequate post-harvest controls[1]. Food products containing aflatoxins end up on the market, affecting the health of consumers. The heterogenous distribution of fungi on agricultural commodities underscores the need for planned sampling in quantifying aflatoxins[2]. The authors highlighted the importance of addressing "how, when and where" the primary sampling should be done for aflatoxins during planning, which is not addressed in many guidelines, although the second step of how-to carryout sampling is described. The timing of sampling is vital as aflatoxin production and degradation continues in all stored kernels with bimodal accumulation-degradation pattern observed in static cultures[3,4]. For quantifying aflatoxins, it becomes necessary to identify the most appropriate sampling point within a product line.

Fungal spores deposit on kernel surfaces in peanuts when exposed to contaminated soil and in oil seeds when exposed to spores in air in storage systems. In smoke cured coconut (copra) kernels, fungi grow when the kernels are inadequately dried and damage occurring during poor handling and storage, exposed to humid environments. Physically damaged surfaces of kernels, moisture distribution within kernels and insect damage lead to conducive conditions for non-uniform localized fungal growth. In a lot, the percentage of contaminated kernels may occur up to 10%, with each kernel carrying different concentrations of

aflatoxins. While several species of fungi producing different mycotoxins may grow on the same copra kernel, aflatoxigenic fungi are predominant at storage. Aflatoxins are produced by strains of *Aspergillus flavus* species and *Aspergillus parasiticus* species, with *Aspergillus nomius* and several other species contributing to a lesser extent[5].

A test sample to examine any parameter in a matrix is expected to represent the lot, from which it is withdrawn. There are standard sampling plans and sample preparation methods for chemical analytes in foods, assuming uniform distribution of the analytes. The assumption, however, is not true for aflatoxins in raw agricultural products[6]. Quantifying aflatoxins in food and feed consists of three steps-sampling, sample preparation and estimation. Of the three steps, sampling leads to highest errors of up to 90% or more in quantifying aflatoxin concentrations in test samples. The contaminations occur in a few kernels of peanuts, corn, cotton seeds, Brazil nuts, walnuts and hazelnuts. Yet they make the whole lot unacceptable[7]. Heterogeneity of aflatoxin distribution on kernel surfaces depends on co-occurrence of toxic and atoxic fungi on kernel surfaces, interspecies inhibition of growth of fungi and mycotoxin production, variations in toxigenicity within the same species of fungi, inhibitory constituents deposited on kernels during processing, resistant proteins in kernels, seed coats, humidity of the environment, moisture content in individual kernels and photodegradation of aflatoxins on exposure to sunlight[7]. Campbell et al.[7] highlighted the importance of designing economical sampling plans based on minimal sample sizes to reduce the cost of sampling in testing aflatoxins. The prohibitive cost is due to heterogenous distribution of aflatoxins in commodities requiring mixing and powdering of sample masses of 10-20 kg from a lot. The current Codex recommendations guide comminuting 20 kg of solid raw material samples to generate a representative test sample[8]. The European Commission guideline requires increment sampling of 100 g building up to 10-30 kg depending on the commodity examined[9]. The Food Standards Agency[10], UK recommends withdrawal of 1 kg. sample for testing for a consignment less than 50 and 10 kg for lots of 20-50 t.

The bimodal pattern of aflatoxin accumulation makes it more challenging to identify when to withdraw a truly representative test sample. Additionally, random sampling to quantify aflatoxins become meaningless due to spatial clustering of fungi on kernels of most commodities[11]. The opportunities of fungal clustering on the surface of copra kernels (274 cm^2 per kernel) are notably high compared to peanut kernel surfaces (6 cm^2 per kernel). When applied to aflatoxin analysis in lots, the general sampling methods may result in processor risks due to overestimates arising from false positive results, or consumer risks due to underestimates arising from false negative results. Both situations are due to heterogeneity of aflatoxin distribution in the matrices leading to discrepancies in sampling[12]. Additionally, unplanned withdrawal of samples from a heterogenous lot may indicate zero or extremely high aflatoxins concentrations when tested. The test results may lead to erroneous reports on the degree of risk associated with the food product. Quantifying aflatoxins in agricultural commodities requires special sampling programs[10]. Whitaker[12] highlighted the importance of standardization of mycotoxin sampling plans of higher performance for the benefit of buyers and sellers. The risks to either party from a poorly representative test sample could be high, though only about 0.1% of the kernels are heavily contaminated in many agricultural commodities[2]. A comparison of reported results examining variance associated in each step of testing agricultural commodities is given in Table 1.

Analysts dry grind kernels during sample preparation to achieve homogeneity. In examining the effect of sampling on deoxynivalenol quantification in wheat grain and wheat flour, high variability was observed for deoxynivalenol in grain sampling, compared to flour sampling. The observation indicates the contribution of particle size of the raw materials on the accuracy of the test results[13]. For quantifying aflatoxins in cocoa, coffee, almond and pistachio, preparation of slurry is reported to give a lower coefficient of variation than comminuting as a dry powder[14]. Adequacy of dry grinding to a fine powder against preparation of a slurry continues to be debated in achieving the degree of homogeneity necessary in quantifying aflatoxins reliably.

Testing animal feeds, which tend to carry relatively homogenous distribution of aflatoxins due to mixing raw materials (unless further contamination occurs) has shown difficulty in eradicating inherent variability associated with sample selection[15]. Kibugu et al.[15] recommended liquid slurry extraction as a better method for quantifying aflatoxins in chicken feed.

The sampling methods applied for aflatoxins by different agencies are reviewed by Donnelly et al.[16]. The authors revealed sampling as the major source of error in aflatoxin quantification in corn. They recommended increased sample size and high frequency of sampling, automated dynamic sampling from moving streams, storage to prevent continuous activity of fungi in samples and the need to be considerate of photodegradation of aflatoxins during handling. Ensuring complete homogenization of the aggregate samples for achieving accuracy in quantifying aflatoxins is therefore necessary. However, the authors indicate absence of clear evidence on effectiveness of the suggested practices but consider deemed the best to reduce errors arising from heterogenic distribution of aflatoxins in the food kernels.

Most studies report heterogeneity of aflatoxins in grains of small kernel size. Coconut oil is extracted from copra, which is dried by smoke curing. Individual copra cups (half

kernels) weighing 180-200 g provides a large surface area for several types of fungi to grow compared to individual kernels of peanut (8 g), corn (0.25-0.30 g), wheat (0.00035 g) and rice (0.02-0.04 g). The information on grains reflects the potential for differences in contamination within individual kernels.

Copra kernels reflect a highly heterogenous distribution of aflatoxins within a single kernel and among kernels. Coconut shell smoke deposited during curing of copra contains organic acids and phenols imparting antioxidants and antimicrobial properties[17,18]. The deposits discourage growth of fungi on smoked surfaces to varying degrees. Since copra kernels have a relatively high surface area and cracks caused by poor handling, they are susceptible to fungi due to their unprotected humid pockets. The distribution of aflatoxins within a single copra kernel tends to be more heterogenous than in individual kernels of grains and other nuts. In edible oils, the sample size needed for testing is certainly smaller, since oil is a homogeneous mixture without aflatoxin production. Coconut oil is among the top 10 edible oils consumed globally. For populations in tropical countries that produce coconuts, it is the major source of lipids of nutritional importance[19]. From a food safety perspective, understanding the changing heterogenicity of aflatoxin distribution in coconut oil production is more important than using contamination data for copra kernels from a toxicological view. The copra cake is a feed constituent for milking animals.

As a result, it is useful to identify the extent of contamination in raw material kernels to eliminate heavily contaminated kernels from the product to reduce the final aflatoxin concentrations. This concept is applied in peanut industries by hand picking or electronic elimination of fungal contaminated kernels. The problems due to heterogeneity discussed above may be applicable to a lesser extent to aflatoxins in edible oils extracted from contaminated oilseeds of smaller size. As a result of the limitations of sampling methods and continuing discussion regarding sampling of agricultural commodities for aflatoxins, a comparison of sampling along steps of coconut processing chain was examined at commercial level in this study. This study provides insight into redistribution of aflatoxin concentrations during mechanical expulsion of coconut oil from smoke cured copra commercially, leading to homogeneity. The study aimed at identifying the most appropriate point of sampling to quantify aflatoxins.

MATERIALS AND METHODS

In sampling of stored copra kernels and copra pieces from the processing lines for testing, different methods were used to withdraw samples and frozen storage of samples pending testing.

Sampling for testing: Samples of coconut kernel products were collected from 44 commercial coconut oil processing mills in Sri Lanka and transported to the laboratory in black sampling bags within 12 hrs of sampling. All samples were stored at - 8°C and subjected to analysis within 7 days. The samples were collected in three rounds over a year from the major coconut processing regions (Western, North-Western and Southern Provinces) in Sri Lanka. The types of samples collected and methods of collection are as follows:

- **Targeted sampling of copra kernels with visible fungal growth pending oil expulsion:** Copra is stored commercially for 3-10 weeks at temperatures of 25-30°C under high humidity (70-80% RH) conditions pending expulsion of oil. The appearance of fungi on copra kernels is heterogenous depending on moisture concentration, the extent of smoke deposited-on the kernel surfaces during kiln drying and damage caused during handling and by insects exposing new surfaces. Individual visibly fungal contaminated cups (half kernels) were withdrawn for testing. Each copra cup was tested separately.
- **Incremental sampling of steamed grated copra pieces of size 2-5 mm:** Copra kernels are steam cooked and grated prior to oil expulsion and are available as heaps for a few hrs. Particles flowing out of the cutter form heaps up to 2 m high and 3 m wide. The oil is extracted from the particles within 24 hrs. The heaps represent a stage in the process flow system. Combined samples of copra pieces of 1-2 kg were obtained from the heaps consisting of 20-200 kg. Each heap was considered a lot consisting of layers of 50 cm each and samples were structured in the ratio of 1: 7: 19: 37:61 from top to bottom in collecting incremental samples. European Commission recommend increment sampling working on aggregates of 1-2 kg for bulk commodity lots to achieve high homogeneity for aflatoxin testing[9].
- **Random sampling of semi processed coconut oil:** Samples were drawn from storage tanks of capacity 500-1000 L. The samples represent filtered oil with no further processing and stored in dark. They tend to be more homogenous due to mixing during continuous

Table 1: Percent errors associated with sampling, sample preparation and quantification of aflatoxins in kernels of several agricultural commodities.

Commodity	Sampling	Sample preparation	Quantifying	References
Shelled corn	77.8	20.5	1.5	Johansson et al.[28]
Peanuts	83	9	8	Whitaker[12]
Almonds	96.2	3.6	0.2	Whitaker et al.[24]
Hazelnuts	99.4	0.4	0.2	Ozay et al.[29]

Table 2: Number of samples tested for aflatoxins

Coconut product	C1	C2	C3	Total
Individual moldy copra kernels	25	35	37	97
Steamed and grated copra pieces	31	26	31	88
Mechanically expelled unrefined coconut oil	44	32	38	114
Copra cake (poonac)	40	34	41	115

C: Collection

Table 3: Aflatoxin B1 (µg/kg) detected in coconut products linked to processing of coconut oil

Coconut kernel products	No.	Positive (%)	Mean	SD	Range	CV
Selected moldy copra cups	97	80	880.8	2335.4	0-20000	2.7
Steamed copra pieces	88	61	75.7	107.9	0-630	1.4
Unrefined coconut oil	114	72	45.4	46.9	0-200	1.0
Copra cake	115	72	88.8	100.0	0-500	1.1

inflow of extracted coconut oil at the top and removal of oil for sale from bottom of the storage tanks. Samples were collected from the flow outlet or dipping a brown sampling bottle halfway down the bulk of oil. Samples were stored in a freezer for one week prior to testing.

- **Random sampling of copra cake (poonac):** Copra cake is the partially caramelized residue left after expulsion of coconut oil from copra. The residue gets heated to temperatures around 120°C during expulsion of oil in screw press mills. As copra cake is a product of grated copra pieces, it is expected to possess similar homogeneity of aflatoxin distribution.

The number of samples tested for aflatoxins from each category during three collections is given in Table 2. The samples were tested in the Aflatoxin Laboratory, Bacteriology Department, University of Peradeniya, Sri Lanka.

Distribution of aflatoxin B1 deep into the copra kernels: The mold contaminated copra kernels with approximate thickness of 1.2 cm were cut into three layers of 4 mm thickness each. The distribution of aflatoxin B1 was tested for combined samples from each layer.

Sample preparation for testing: Frozen field samples of weight 1-2 kg were comminuted in a laboratory sponge mill No. 3, to obtain a homogenous test sample. The resulting powders carried the following particle size distribution:

10 mesh (2 mm) retained : 10%
10-20 mesh : 50%
20 mesh (0.84 mm) through : 40%

In designing aflatoxin sampling plans for peanuts, use of hammer mill to reduce the particle size to pass through sieve number 14 (3.1 mm aperture) was suggested[1]. The report recommends grinding peanuts to pass through a 2 mm sieve.

Analytical samples of 25 g were blended with hexane (100 mL) and 55% aqueous methanol (225 mL) with 0.5 g sodium chloride for 3 min to make a slurry. Slurry was filtered under suction[20]. The aqueous methanol fraction of the filtrate was separated from hexane in a separatory funnel and aflatoxins extracted into chloroform. The chloroform fractions were dried using anhydrous sodium sulphate and concentrated under low pressure and temperature of 60°C in a rotary evaporator under gentle suction. All extractions were done in duplicate.

The estimation was done by comparing fluorescence of the aflatoxin spots of samples (4 volumes) against standard aflatoxin samples (4 volumes) on TLC (Silica Gel G-Merck)) developed using solvent systems methanol-chloroform (3:97) and acetone-chloroform (1:9). Estimations were done based on scanning densitometry for fluorescence at 365 nm. Minimum detection limit of aflatoxin B1 was 20 µg/kg by the method.

Glassware was cleaned with chromic acid and grinding mill with acetone swabs to prevent cross contamination between sample preparation and testing. Microsoft Excel program was used to calculate mean, standard deviation and coefficient of variation.

RESULTS AND DISCUSSION

Properties of copra favoring aflatoxin contamination: Coconut kernels contain nutrients and moisture that facilitate fungus colonization. The soft, unprotected inner kernel surfaces are highly vulnerable to fungal colonization, except for protection by antimicrobials deposited during smoke curing and moisture reduction. Each copra cup (half kernel) provides a larger surface area than individual grains for fungal growth. Average inner diameter of a copra cup is 90 mm and 274 cm^2 is the average surface area[21]. The fungi grow mostly on the soft inner surface of the copra kernels. The outer surface of copra kernels is protected by a hard brown testa which is rich in antioxidant phenolics[22]. In 12 mm thick copra kernels, the inner (colonized side) 8 mm of copra kernels were found to contain 90% aflatoxin B1, whereas the outer 4 mm near the testa contained 10% aflatoxin B1. The fungal hyphae tend to penetrate the soft kernel and aflatoxins are deposited inside the kernels. A previous study has shown that short and medium chain fatty acids are used by fungi to accumulate aflatoxin in synthetic

microbiological media containing aqueous extracts of fresh coconuts[23]. It is easy for aflatoxigenic fungi to grow on copra kernels due to the nutrients they contain.

Heterogeneity of aflatoxins distribution in commercial copra products: Because of the heterogeneity of aflatoxin accumulation, sampling practices in different agricultural commodities cause 77-99% errors in estimations (Table 1). As a result of commodity-related dimensions and resistance factors, the heterogeneity is linked to the spatial distribution of aflatoxins in individual kernels.

Table 3 shows the aflatoxin B1 concentrations detected in the contaminated raw material (copra), its products during processing (copra pieces) and the end products, unrefined coconut oil and copra cake.

In this study, the heterogeneity of fungal colonization and aflatoxin production is viewed as of two distinct characters associated with processing. Firstly, the targeted fungal infested copra kernels are expected to carry heavy aflatoxin concentrations with highly heterogenous distribution. Individual kernels infested with fungal growth contained aflatoxins in 80% of cases. Twenty percent of fungi infested copra kernels did not contain aflatoxins, suggesting the presence of non-aflatoxigenic fungi. Slide cultures on fungi picked from the kernels showed Penicillium species, *Aspergillus ochraceous* and Mucor species in addition to *Aspergillus flavus*/parasiticus. Aflatoxigenic fungi may be prevented from colonizing kernels at least in part by non-aflatoxigenic fungi. There was a coefficient of variation (CV) of 2.6 in the aflatoxin estimates for the targeted kernels, indicating that the distribution of aflatoxin was highly heterogeneous. It is less useful to quantify aflatoxins in copra kernels but is indicative of the risks associated with coconut oil derived from coconut kernels.

The second category is a random sample of steamed copra pieces, unrefined coconut oil and copra cake, which are expected to show relatively homogenous distribution of aflatoxins. Copra cups are steamed and cut into small pieces followed by delivering into a heap. This results in a mixed product. The samples tested in this category were from aggregates of increment samples collected from stratified layers in order to achieve high sample homogeneity. The test results showed a cv of 1.4, which still is in the high side. However, the decrease of cv from 2.6 in targeted kernels to 1.4 in random samples (mixed pieces) reflects a remarkable increase in homogeneity of aflatoxin distribution arising from reducing the particle size. Whitaker et al.[24] reported a cv of 1.3 for sampling of almond kernels. Copra cut pieces analyzed in the current study are of comparable dimensions to almond kernels, indicating similar kernel sizes with the cv of 1.4. Whitaker et al[25] reported a cv of 0.063 for deoxynivalenol in wheat grains and relates the low cv to small size of the wheat grains (1/30 of peanuts). In wheat, kernel count is 30,000 per kg against 5 per kg in copra. In copra pieces a relatively high cv is expected as it is produced from a mixture of moldy and mold free kernels.

The extracted coconut oil is assumed to be homogenous as the oil get mixed continuously in the pipelines and during delivery to the storage tanks. The cv for aflatoxin B1 in coconut oil is 1.0 which is more acceptable statistically for a product generated by a biological material, with limited control on fungal infestations. A cv of 1.0 is considered satisfactory for natural microbiological contamination of foods carrying non-motile microorganisms delivering the toxins to surroundings of the hyphae. As expected, copra cake, the residue after extraction of oil, reflects a remarkably close cv of 1.1 to that of coconut oil, though further mixing does not occur among copra cake particles. In collecting copra cake random sampling was done. The level of aflatoxin in copra cake is a result of mixing up that occurs when the copra pieces are transferred from the heaps to the oil expeller using buckets. The cv for coconut oil and copra meal remains close, indicating low homogenization of aflatoxin distribution in the coconut oil storage tanks. Whitaker[12] highlighted the importance of grinding kernels and taking subsamples from ground material in order to get reliable results for mycotoxins.

When coconut oil is produced from heterogeneously contaminated copra, there is considerable homogenization of aflatoxin distribution as a result of reducing the particle size from cups to pieces of 3-5 mm. Due to the high levels of aflatoxin in copra kernels, coconut oil does not meet regulatory standards. This observation indicates the need to eliminate visibly fungal infested copra kernels manually or by other means. Due to the visible contamination, manual elimination of contaminated kernels before cutting into pieces seems necessary and possible. In order to prevent post-harvest contamination in expelling oil for edible purposes, it would be more rational to reduce the storage period of copra kernels.

Reporting aflatoxins in agricultural produce and kernel dimensions: According to Kumphanda et al.[26], a meta-analysis of publications on aflatoxins on sample size and sample preparation of maize found that 50% of publications did not mention sampling methods and two-thirds of publications did not mention the grinding equipment used and particle sizes achieved by sieving in sample preparation. It has been observed in many publications over the last three decades that test samples are becoming smaller. The low volumes of test samples raise doubts about the representativeness of the results. Compared with smaller grains, copra shows a highly heterogeneous distribution of aflatoxin due to its large kernel size. As a result of quantifying aflatoxins in coconut oil, regulatory actions

require small sample size of copra. Heterogeneity may change during mixing of raw materials during transport and during processing. This concept applies to all edible oil extraction industries to different degrees.

Best sampling points in process line of contaminated commodities: It may be possible to understand the risk prior to consumption by identifying the optimal sampling point during processing of contaminated agricultural commodities. To achieve this, we must understand changes in aflatoxins distribution at each processing step, as well as contamination patterns in raw materials. Most edible oils contain aflatoxins originating from oil seeds. The oils continue to carry aflatoxins unless they are chemically refined. Chemical refining of edible oils is not practiced in less developed countries. Currently, there is increasing consumer concern on the safety and nutritional quality of refined foods including edible oils. By understanding how aflatoxin concentrations are reduced with processing steps, it would be possible to reduce aflatoxins in foods and edible oils below regulatory limits.

A study on vegetable oils in Sri Lanka has indicated 1.8-60.9 µg/kg of aflatoxin B1 in market coconut oils compared to less than 0.8 µg/kg in eight other types of edible vegetable oils. The other types of vegetable oils are refined oils, in contrast to unrefined coconut oil used in the country. In the unrefined coconut oils, 38% samples contained aflatoxins[27]. In this study, the values reported for commercial coconut oil show comparable evidence of reduction of aflatoxins in oils during processing (Table 3).

Based on changes in heterogeneity of aflatoxin distribution in the raw material copra and the processed final product coconut oil, the analysis of coconut oil appears to provide more meaningful results for use in regulatory decisions with least risk to producer or consumer.

CONCLUSION

Testing fungus-infested copra kernels do not provide a true picture of aflatoxin contamination in a lot, as some of the fungus infested kernels did not contain aflatoxins. A 50% reduction in the coefficient of variation of aflatoxins in grated copra from the processing line indicates partial homogenization of aflatoxin distribution. A comparison between the cv of targeted kernels and coconut oil shows 75% reduction in coefficient of variation indicating that testing oil is the best option for testing of aflatoxins for public health. As copra is processed into coconut oil, the aflatoxin distribution in test samples becomes more homogeneous.

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