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Aflatoxin Prevalence Data Collection

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SAMPLING FRAMEWORK & METHODOLOGY

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INTRODUCTION

Information is fragmented on the prevalence of aflatoxins, a variety of mycotoxin, in the maize value chain in Kenya and in the groundnut value chain in Mali. The purpose of this study is to generate a consistent database of aflatoxin prevalence along the maize and groundnut value chains through systematic sampling, and to identify critical points where intervention strategies are likely to have the greatest impact.

It is important to remember that aflatoxin-contaminated units are not homogeneously distributed throughout a plot: A few units are likely to be highly contaminated (mycotoxin clusters), while most of the grains are mycotoxin-free. Collecting samples only from the highly contaminated grains or from the mycotoxin-free ones will provide inaccurate final results. Therefore, proper sampling is one of the most crucial elements of addressing and managing aflatoxin contamination of food.

The overall objective of good sampling is to provide reliable samples for analysis that can represent the basis for ‘‘fit for purpose’’ investigations. Samples should be randomly gathered from many incremental samples, whether from the field or from grain/groundnut in bulk, in order for the analysis to be representative of the whole lot.

Meaningful sampling involves two key steps:

1. Primary sampling: the process of ‘‘statistically’’ locating the sites (populations) from which samples should be taken (that is, making the decision of ‘‘why, where, and when’’ to collect the samples)
2. Secondary sampling: the process of establishing how samples should be collected in order to be representative of the lot under investigation

A good sampling plan should cover the full range of environmental variables, including multiple elevations and diverse weather conditions.

PRIMARY SAMPLING FRAMEWORK: MAIZE IN KENYA

Aflatoxin contamination of maize has been reported in many districts in eastern Kenya, including Machakos, Makueni, Kitui, and Mbeere, as well as in Thika District (central Kenya). For the purposes of this project, a stratified sampling approach will be followed, with sampling both in high-risk areas (where cases of acute aflatoxin poisoning have been reported) and in low-risk areas (areas with high maize consumption but no reported cases of acute aflatoxin poisoning). Low-risk areas include: Western Province (Kakamega, Bungoma, and Migori Districts); Central Province (Nyeri, Kiambu, Nyandarua, and Muranga Districts); Rift Valley Province (Trans Nzoia, Uasin Gishu, Molo, and Nakuru Districts); Nyanza Province (Kisii, Migori, Siaya, and Kisumu Districts); and Coast Province (Kilifi and Taita Taveta Districts).

Sampling will be conducted in two districts of eastern Kenya and at least one district of western Kenya (see Appendix 1, Map 1). The two districts of eastern Kenya represent the high-risk areas, while the districts of western Kenya represent the low-risk areas. Farmers and markets to be included in the samples will be located along transects in each of these areas, so as to capture a range of agroecological zones. Thus, sampling will be undertaken in:

1. Nyanza Province in western Kenya: from Kisii to Homa Bay (high to low elevations). This transect will be linked to the CIMMYT storage pest project, which will be collecting samples from the same area.
2. Upper eastern Kenya in Eastern Province: Embu to Mbeere, representing different agroecological areas that run from high to low elevations.
3. Lower eastern Kenya in Eastern Province: to include the districts of Machakos and Makueni.

There are six agroecological zones in the Kisii to Homa Bay transect (with at least three districts along the Kisii–Homa Bay transect), and seven zones in the Embu–Mbeere transect. Ideally, 30 samples should be collected from each agroecological zone.

Maize: Sampling schedule

Sampling will be performed periodically along the value chain—production, transportation (grain, wholesale, and maize traders), storage, and processing (*posho* millers, etc.)—in order to assess changes in aflatoxin prevalence over time and along the value chain. Grain traders and wholesalers will be identified and grain will be sampled after offloading at storage facilities. It is best to sample at the storage point as grain is not held long enough in transport.

Sampling at production point

Samples will be collected from:

1. At pre-harvest in the field, after physiological maturity (about a week before harvest), and
2. After the post-harvest activities are completed (approximately 15–30 days later).

Sampling at storage and processing points

Samples will be collected from:

1. Farms (farmer's own storage structure): samples will initially be collected between 15 and 25 days post-harvest, and then monthly until the next cropping season, or until grain is depleted
2. Retailers
3. Processors: small-scale (*posho*) millers, large-scale millers, and food and feed processors
4. Warehouses: (a) government; (b) private

The same sampling plan will be followed for all storage points: farm level, private, and government.

Sampling at market

Market sampling will be done every month (until next harvest or until grain is depleted), depending on the availability of grain. Sampling will take place at four points in the marketing process: (a) assemblers, (b) wholesalers, (c) retailers, (d) consumers of products. Depending on the volume stored, one or more samples will be collected from each actor, so that samples are representative of the grain being stored. One market in each sublocation will be selected and samples will be collected from at least five traders in the market. Samples will be based on the particular batch supplied to the trader, as traders may have the same batch for some time. Alternatively, samples can be drawn every month from the same lot.

Maize: Data collection guidelines

Tables 1 and 2 summarize in detail the type of data and number of samples to be collected at each level of the maize value chain in Kenya. We will collect data separately for *traditional* and *improved* methods of production and storage, where traditional storage refers to a farmer's own structures and improved storage refers to the use of modern granaries, metal silos, or hermetically sealed bags.

Table 1. Types of data to be collected at different levels of Kenya maize value chain, traditional production and storage

Actor	Location	Data type	Approximate sample size (number of actors)
Producers	Field plot	Farmer's name; name of district, division, location, and sublocation, village; agroecological zone, GPS coordinates; maize variety, % moisture content (MC); how grain is dried	420 (14 agroecological zones (AEZ) in 3 districts * 30 samples per AEZ)
Producers	Storage (farmer's storage structures)	Socioeconomic information: Total amount of produce; length of storage period; type of storage structure; quantity of produce sold; quantity of grain bought to supplement own stock, and source of purchased grain	2940 (14 AEZs * 30 samples per AEZ * 7 months of storage time)
Traders and wholesalers, retailers, open air vendors, etc.	Storage	Source of grain, length of storage period; how grain is handled; % MC; types of storage facilities	36 (2 types (govt. and private) * 3 districts * 6 months minimum storage period)
Processors	Storage	Source of grain; type of processing; how processed products are stored	84 (1 processor per AEZ * 14 AEZ * 6 months of storage)
Processors	Processed products	Type of processed products; storage conditions. Information about other processed products sold should be collected at retail outlet (e.g., flour, dehulled maize, etc.)	
Total for TRADITIONAL PRODUCTION AND STORAGE OF MAIZE			**3480 samples**

Table 2. Types of data to be collected at different levels of Kenya maize value chain (Improved production and storage)

Actor	Location	Data type	Sample size (number of actors)
Producer	Storage (granary)	Produce stored; length of storage period; type of storage structure	540 (3 districts * 30 samples per district * 6 months storage period)1
Producer	Storage (metal silos, hermetically sealed bags)	Type of storage structure; pre-storage treatment/handling of produce; chemicals used to treat grain during storage, etc.	324 (18 samples * 3 sites (one per district) * 6 months storage period)2
Total for IMPROVED PRODUCTION AND STORAGE OF MAIZE			**About 864 samples1**
GRAND TOTAL OF MAIZE SAMPLES			**4344 samples1**

Notes:

[1]The total number of samples depends on grain availability.

[2] Duration of storage is variable and is dependent on the next harvest; in most cases it is less than 6 months, as rainfall is bimodal (two seasons per year).

PRIMARY SAMPLING FRAMEWORK: GROUNDNUTS IN MALI

Three locations in Mali were selected for aflatoxin testing in groundnuts: Kita and Kayes Cercles[1] in Kayes Region, and Kolokani Cercle in Koulikoro Region. All locations are in the western part of the country. More than 70 percent of Mali's national groundnut production comes from these three zones. In each location, groundnut samples will be collected at several points along the groundnut value chain: farms; storage facilities of farmers, collectors, and processors; rural markets; urban and semi-urban markets; and seed distributors. Tables 3 and 4 summarize the type of data to be collected at each level of the value chain.

Villages were selected on the basis of agroecological zone and road accessibility. An effort will be made to ensure spatial distribution by selecting villages at least three kilometers (km) apart from each other. Three farmers will be selected in each village. (See Appendix 1, Maps 2, 3, and 4, showing selected villages for the three areas.)

Groundnuts: Sampling schedule

At each level of the value chain, a specific approach will be followed for collecting samples. At the level of production (farmers' level), the same approach for sampling will be followed in both traditional and improved groundnut production and storage types. Table 5 summarizes projected approaches for groundnut sampling.

Table 3. Sampling approach for different actors along the groundnut value chain

Actor/Location	Period	Sampling scheme
Producers (Farmer's field)	At harvest	5–10 samples per farmer, to be ground and mixed (1 kg total)
Producers (Granaries)	Day of storage	5–10 samples (500 grams total)
	15 days in storage	5–10 samples (500 grams total)
	30 days in storage	5–10 samples (500 grams total)
	Then monthly	5–10 samples (500 grams total)
Traders, wholesalers, processors	Storage room Once	For N < 10 bags stored: sample from each bag (up to 10 bags) For N > 10 bags: sample from M=Sup [$\sqrt{N}+1$, 10] where M = maximum number of samples (starting from a minimum of 10), and N = the number of bags to be sampled from.
Rural markets	Market place Once a month	20 samples (10 seed and 10 processed product)
Semi-urban and urban markets	Market place Once a month	20 samples (10 seed and 10 processed product)

[1] A cercle is an administrative unit, equivalent to a district in Kenya; a region is an administrative unit equivalent to a province in Kenya.

Groundnuts: Data collection guidelines

Tables 3 and 4 summarize in detail the type of data and number of samples to be collected at each level of the groundnut value chain in Mali. We will collect data separately for *traditional* and *improved* methods of production and storage. Improved production pre-harvest entails the use of improved seed varieties and spreading lime, manure and crop residues. Improved harvesting methods include shelling the nuts immediately upon harvesting, and drying the groundnuts more rapidly by ensuring greater exposure to the sun. Improved storage includes the use of insecticides and storing groundnuts in a room linked to the cooking area so that smoke from the kitchen is captured in the granary.

Table 4. Types of data to be collected at different levels of Mali groundnut value chain (Traditional production and storage)

Actor	Location	Data type	Sample size (number of actors)
Producers	Field plot	Land preparation; variety; date of planting; use of manure; use of mineral fertilizers; use of pesticides / insecticides; weeding; date of harvest; harvesting technique (drying, winnowing, transport, threshing or degoussage); product quality, etc.	270 (3 cercles * 30 villages per cercle * 3 farmers (plots) per village)
Producers	Storage (Granaries)	Health status; type of granary; granary status; conditioning; number of months in the granary	2700 (3 cercles * 30 villages per cercle * 1 farmer per village * 10 samples each year)
Traders and wholesalers	Storage	Source of seed; health status; type of storage (store room); storage room status; conditioning	150 (3 cercles * 5 traders per cercle * 10 samples each year)
Wholesalers	Storage (trade)	Source of seed; health status; type of storage (store room); storage room status; conditioning	15 (3 cercles * 1 wholesaler per cercle * 5 samples each year)
Processors	Storage	Pre-processing operation; processing technology; storage technology	45 (3 cercles * 5 processors per cercle * 3 samples/year/ processor
Processors	Processed products	Pre-processing operation; type of processed product (oil, paste, etc.…); processing technology	120 samples = 2 cercles * 10 samples / cercle * 3 times/year * 2 years
Rural markets	Seed	Source; variety; price; visual quality; type of packaging	360 (3 cercles * 10 samples per cercle * 12 months)
Rural markets	Processed products	Source of seed; type of processed product; processing technology	360 (3 cercles * 10 samples per cercle * 12 months)
Semi-urban and urban markets	Seed	Source; variety; price; visual quality; type of packaging; transport; duration in storage (months); storage status	360 (3 cercles * 10 samples per cercle * 12 months)
Semi-urban and urban markets	Processed products	Source of seed; type of processed product; processing technology	360 (3 cercles * 10 samples per cercle * 12 months)
Total for TRADITIONAL PRODUCTION AND STORAGE OF GROUNDNUTS			**4740 samples**

Table 5. Types of data to be collected at different levels of Mali groundnut value chain (Improved production and storage)

No	Actor	Location	Data type	Sample size (number of actors)
	Producer	Field plot (led by IER[1])	Land preparation; variety; date of planting; use of manure; use of mineral fertilizers; use of pesticides / insecticides; weeding; date of harvest; harvesting technique (drying, winnowing, transport, threshing or "degoussage"), product quality, etc.	**48** (2 cercles * 1 variety * 12 fields per cercle + (1 cercle * 2 varieties * 12 fields))
	Producer	Storage in granaries (led by IER)	Health status; type of granary; granary status; conditioning; number of months in the granary	**126** (3 cercles * 3 granaries per cercle * 14 samples per granary)
	Producer	Storage in double bags (led by IER)	Source of seed; health status; type of storage (store room); storage room status; conditioning	**504** (3 cercles * 3 farmers per cercle * 2 bags per farmer * 14 samples each year * 2 years)
Total for IMPROVED PRODUCTION AND STORAGE OF GROUNDNUTS				**678 samples**
GRAND TOTAL OF GROUNDNUT SAMPLES				**5418 samples**

[1]Institut d'Economie Rurale

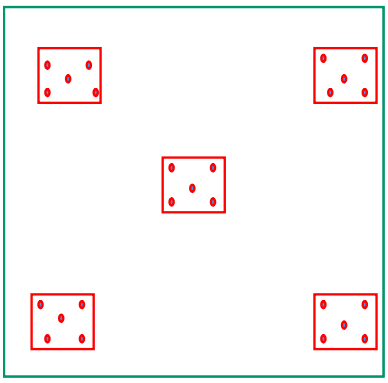
SECONDARY SAMPLING FRAMEWORK FOR MAIZE AND GROUNDNUTS

At each level of the value chain, a specified approach will be followed for collecting samples.

Pre-harvest sampling

For maize, five sample stations, each measuring 5 rows by 5 m long, will be identified in each plot. Five cobs will be collected from five randomly selected maize plants at each sampling station (see Figure 1). A total of about 25 cobs will be collected from each field, hand-shelled, and thoroughly mixed to form a composite sample, of which a 1 kg sample will be randomly collected and sun-dried to about 13 percent moisture. For groundnuts, five squares will be indentified within a field. Five samples of at least 100g will be collected and shelled by hand from each square. These samples will be combined to make 1 *lot,* from which 1kg will be selected randomly and sun-dried to about 13 percent moisture.

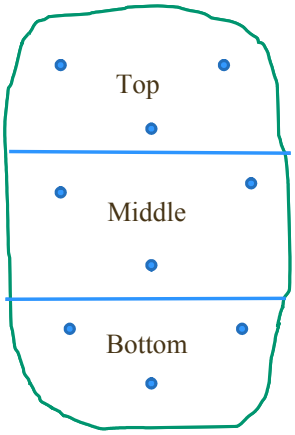
Figure 1. Sampling from the field – at harvest



Post-harvest sampling from storage

When sampling from storage structures (static lots—bins, sacks, or containers,), multiple probing should be done. Small numbers of samples are collected from different areas of a container and then mixed to produce a representative sample. Samples will be taken with a probe, at three levels: top, middle, and bottom (Figure 2). At each level, samples of 1 kg are taken randomly and then mixed. Then, 1 kg is drawn at random from the mix.

Figure 2. Post-harvest sampling from storage structure (bag, silo, or other)



All groundnut samples collected will be sent to the ICRISAT station in Bamako for aflatoxin detection. In Kenya, sundried samples will be transported to the laboratory, milled, transported to ICRISAT in Nairobi, and kept at 4°C until processed. Staff will be trained in sampling procedures and labeling.

AFLATOXIN ANALYSIS PROTOCOLS

All samples will be analyzed using an enzyme-linked immunosorbent assay (ELISA) method, using ICRISAT's in-house protocol. The ELISA test for aflatoxin is published by ICRISAT (see Annex B). Samples will be tested at ICRISAT facilities located in Nairobi, Kenya and in Bamako, Mali.

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APPENDIX 1. TABLES AND MAPS SHOWING SAMPLING TIMETABLE AND SITES

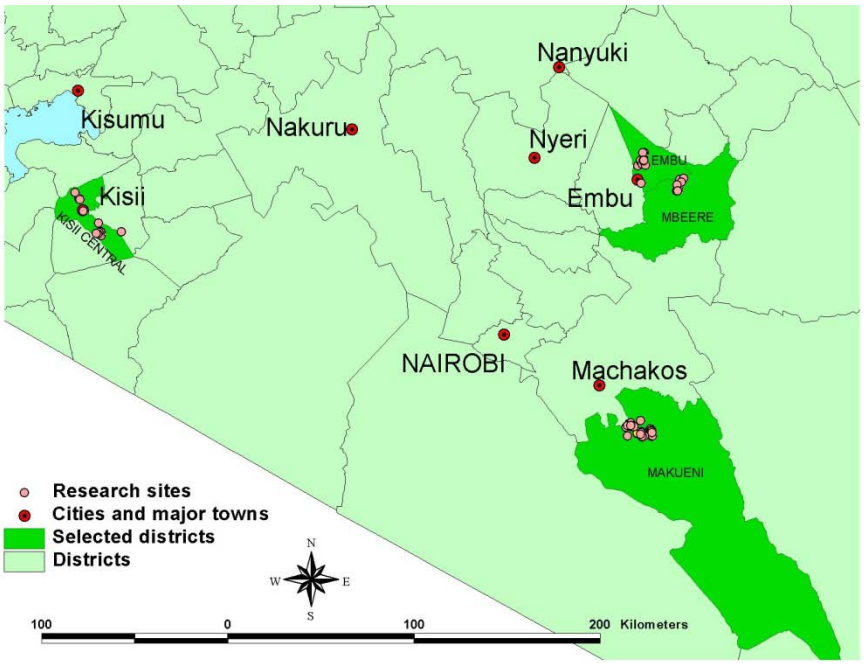
Table A1. Proposed sampling periods in Kenya

District where samples will be collected	Number of samples per region at each sampling	Sampling at Harvest
Upper eastern Kenya: Embu to Mbeere	6 agroecological zones at 30 samples per zone = 180 samples	July–August February–March
Lower eastern Kenya: Makueni district only	30 samples	August–September February–March
Western Kenya: Kisii to Homa Bay (Bungoma)	7 agroecological zones at 30 samples per zone = 210 samples	November–December July-August–September

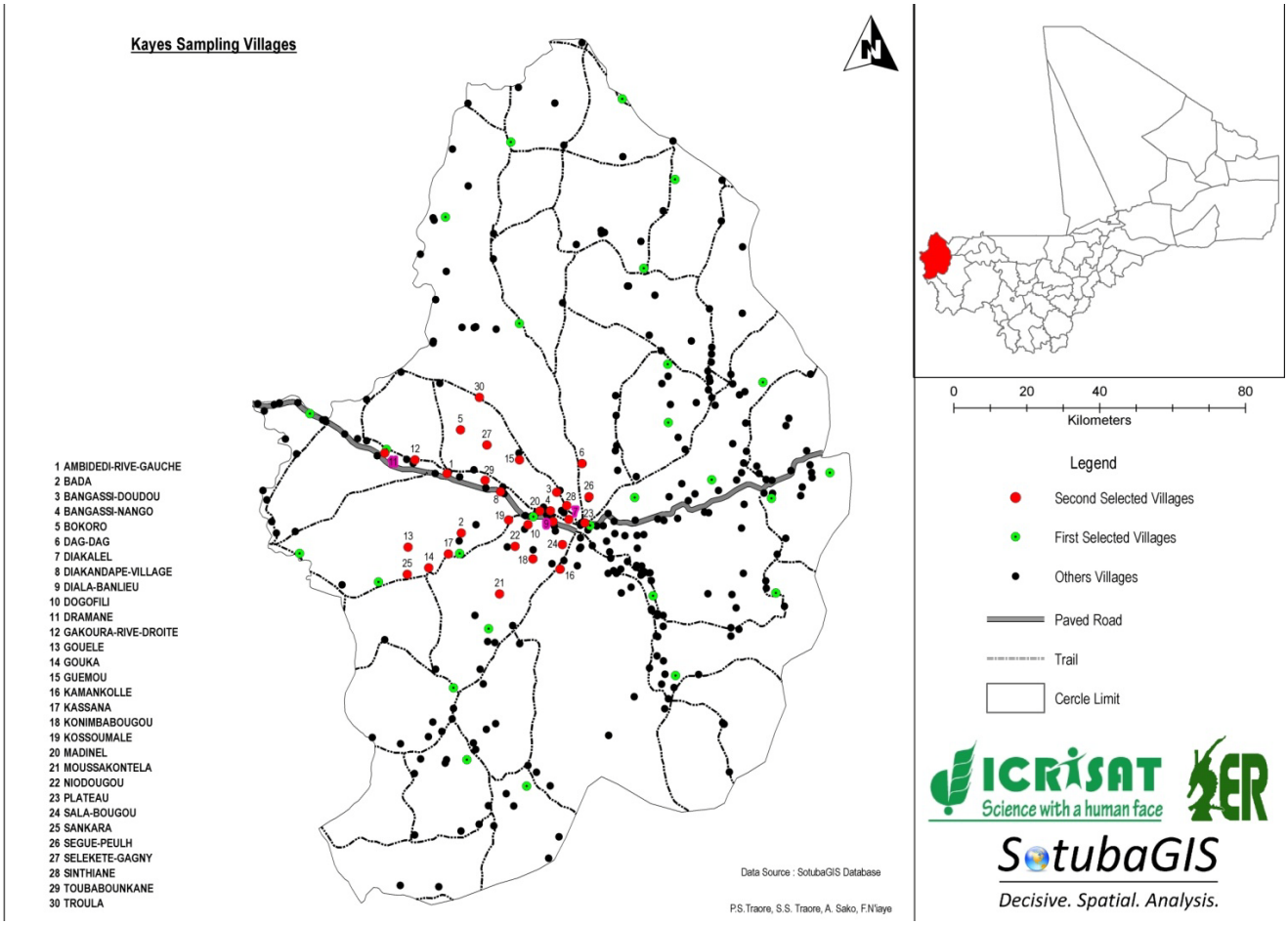
Table A2. Timeframe for prevalence data collection activities in Mali

Actor	Sampling sites	Starting date	Samples
Producers	Farmer's field	November 2009	180
	Granaries	November 2009	1800
	Improved granaries	November 2009	126
Traders	Storage room	November 2009	100
Wholesalers	Storage room	November 2009	10
Processors in storage	Storage room	November 2009	90
Processors	Processed products	November 2009	120
Rural markets	Market place	November 2009	480
Semi-urban and urban markets (Kita, Kayes)	Market place	November 2009	480
Total			**3386**

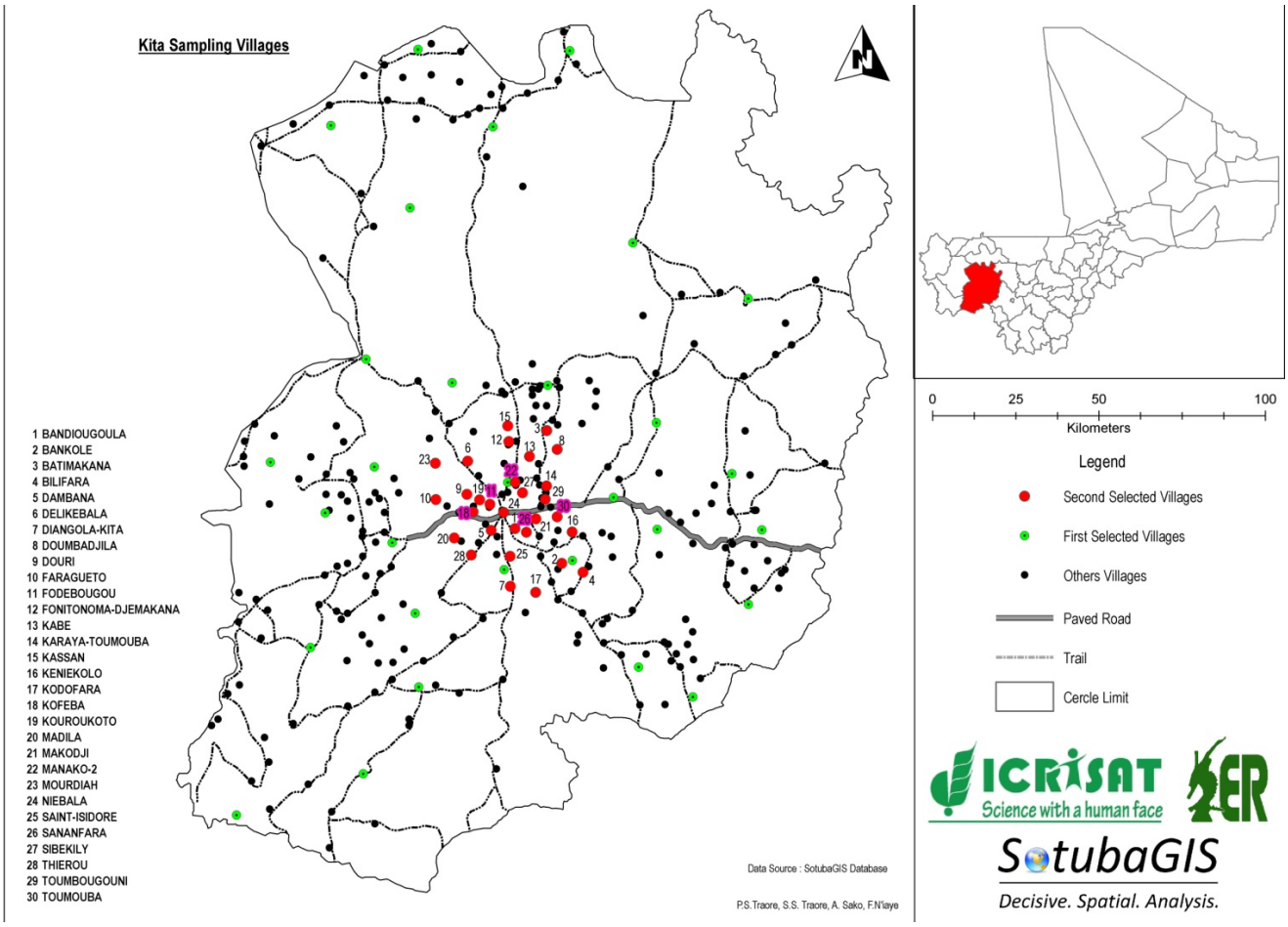
Map 1. Distribution and location of maize sampling sites in Kenya



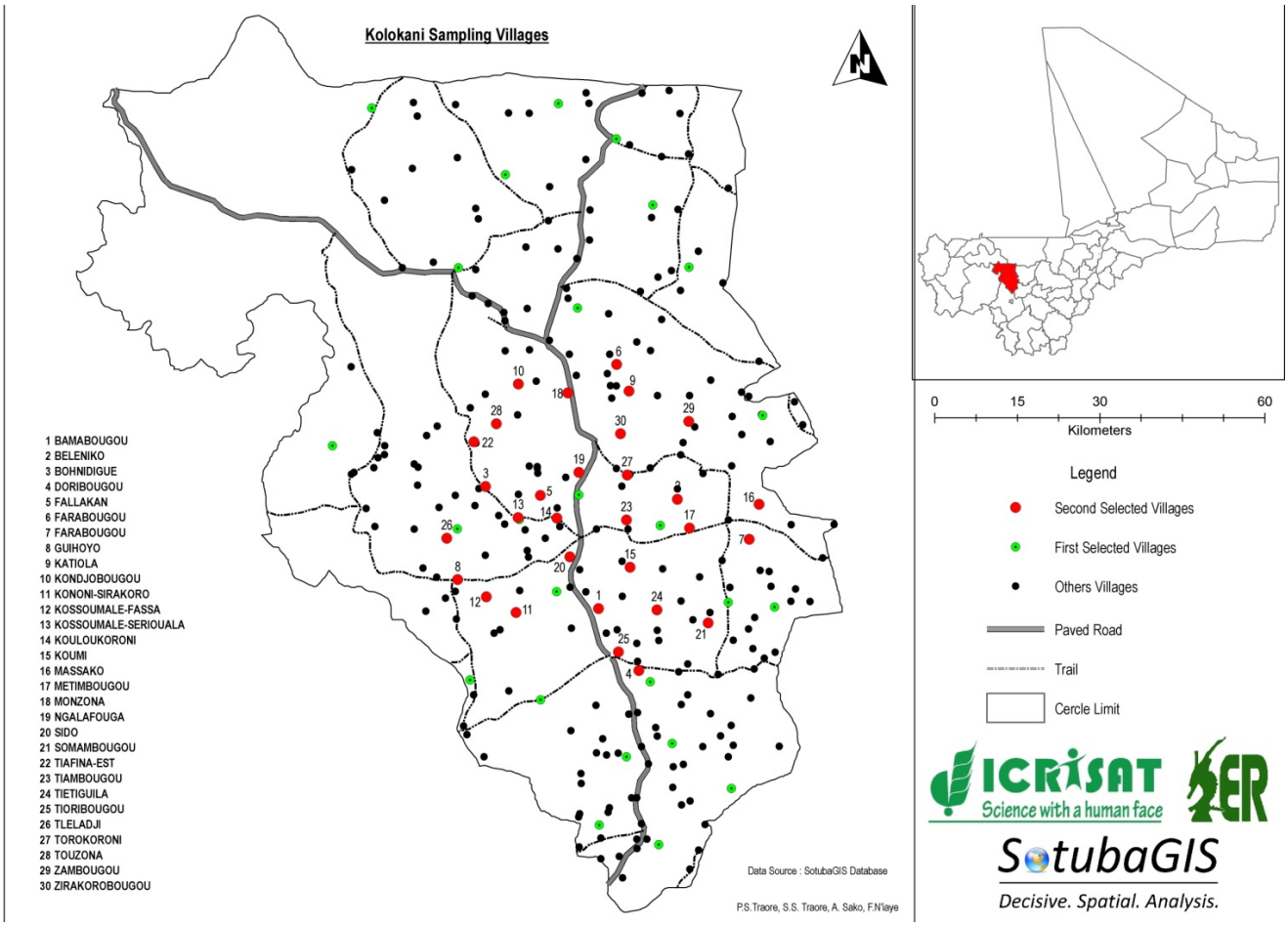
Map 2. Participating villages in groundnut samples collection in Kayes Cercle



Map 3. Participating villages in groundnut samples collection in Kita Cercle



Map 4. Participating villages in groundnut samples collection in Kolokani Cercle



APPENDIX 2. ELISA TESTING PROTOCOL[2]

Sample extraction (e.g. groundnut): Powder 100 g of groundnut kernels using a blender. Take 20 gm of this powder, add 100 ml 70% methanol (v/v-70 ml absolute methanol in 30 ml distilled water) containing 0.5% KCl and blend them until the mixture is thoroughly homogenized. Transfer the extract to a conical flask, seal it with parafilm and shake it for 30 minutes at 300 rpm in a mechanical shaker. Filter the extract through Whatman No. 4 filter paper, and store the filtrate at 4°C till needed for analysis. In the same way, prepare a toxin-free sample (healthy groundnut - HGN) extract, which will be used for dilution of standards as well as a negative control.

ELISA test: ICRISAT developed two types of ELISAs for the analysis of aflatoxins: (i) indirect competitive ELISA and (ii) direct competitive ELISA. Both types are heterogeneous competitive assays that involve the separation of free (un-reacted) toxin in liquid phase from the bound toxin in solid phase. The basic principle of ELISA lies in trapping the antigen on a solid surface, or capturing the antigen with specific antibodies, and probing with specific immunoglobulins carrying an enzyme label. The enzyme, retained in the case of a positive reaction is detected by adding a suitable substrate. The enzyme converts the substrate to a product that can be easily recognized by its color. Moreover, both ELISAs were developed using alkaline phosphatase, pencillinase, horseradish peroxidase enzyme systems separately, and they do not significantly differ from each other.

Equipment: ELISA reader, incubator, orbital shaker, ELISA plate shaker, a set of micro-pipettes including 12 channel one, balance, pH meter, fume hood, vortex mix, waring blender with mini jars, ELISA plates (Nunc, Maxisorp).

Preparation of reagents

Aflatoxin B1-BSA conjugate (AFB1-BSA): Dissolve 1 mg AFB1-BSA (Sigma 6655) in 1 ml sterile distilled water.

Carbonate buffer (coating buffer): Na2CO3 1.59 g, NaHCO3 2.93 g, distilled water 1.0 L, pH of buffer should be 9.6 (No need to adjust the pH).

Phosphate buffered saline with Tween (PBS T): Na2HP04 2.38 g, KH2P04 0.4 g, KCl 0.4 g, NaCl 16.0 g, Tween 20: 1 ml, distilled water 2 L.

PBST-BSA: Dissolve 200 mg bovine serum albumin (BSA) (Sigma A 6793) in 100 ml PBS-T.

Substrate buffer for alkaline phosphatase system: It is preferable to use the p nitrophenyl phosphate (pNPP) chemical in tablet form (available in 5, 15 or 20 mg tablets). Prepare 10% diethanolamine (v/v) in distilled water, adjust pH to 9.8 with concentrated HCl. Prepare 0.5 mg ml-1 pNPP in 10% diethanolamine, pH 9.8 (30 ml solution is required for each 15 mg tablet).

Sample analysis design: Aflatoxin standards and samples should be placed in the plate as given in Figure 1. Standards and samples should be tested in two wells. Occasionally the border wells give non-specific backgrounds, so it is advisable to avoid the border rows. In both ELISAs, the plate should be incubated at 37°C preferably on an ELISA plate shaker.

[2] Source: Waliyar, Reddy, and Kumar 2009.

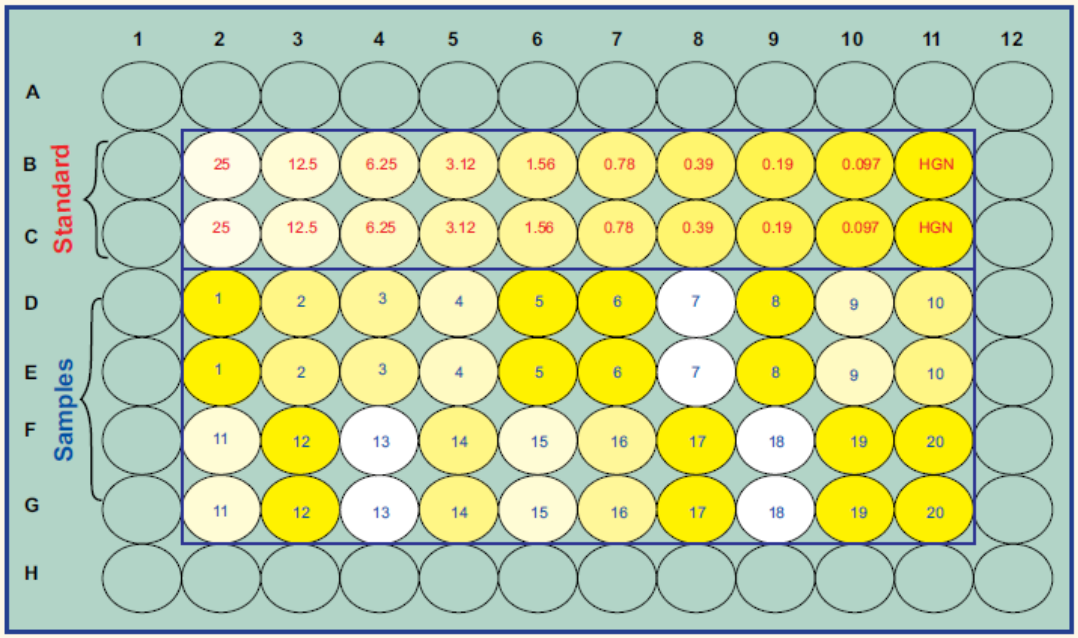
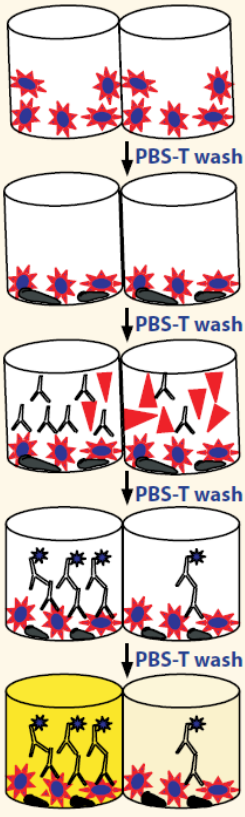


Figure1. ELISA plate design with end color development.

Indirect competitive ELISA protocol



Coating: Prepare AFB1-BSA in carbonate buffer (100 ng/ml) and add 150 µl in each well. Incubate the plate at 37°C for 1 hour.

PBS-T wash

Blocking: Add PBST-BSA and incubate at 37°C for 30 minutes.

PBS-T wash

Competition: Prepare AFB1 standards (25-0.097 ng/ml) in 10% toxin-free extract or PBST-BSA with 7% methanol and add 100 µl/well in rows B-C of the plate. Similarly add 100 µl diluted sample extract (1:10 in PBST-BSA) in rows D-E. Add 50 µl diluted antiserum (1:6000 in PBST-BSA) to all the wells and incubate at 37°C for 1 hour.

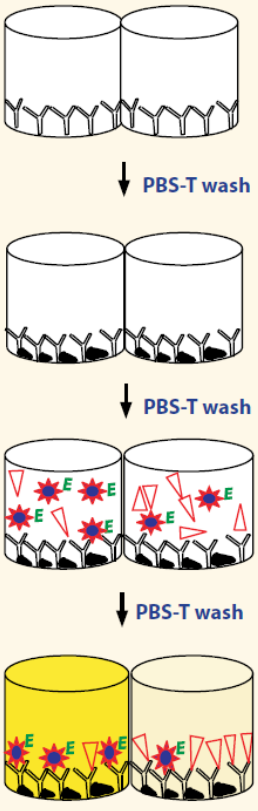
PBS-T wash

Enzyme conjugate: Add 150 µl anti-rabbit-IgG-ALP (1:4000 in PBST-BSA) to all wells and incubate at 37°C for 1 hour.

PBS-T wash

Substrate: Add pNPP prepared in 10% diethanolamine. Color develops in 20 min. Read the plate in ELISA reader at 405 nm.

Direct competitive ELISA protocol



Coating: Dilute the antiserum (1:2000) in carbonate buffer and add 150 µl in all the wells. Incubate the plate at 37°C for 1 hour.

PBS-T wash

Blocking: Add PBST-BSA and incubate at 37°C for 30 minutes.

PBS-T wash

Competition: Prepare AFB1 standards (25-0.097 ng/ml) in 10% toxin-free extract or PBST-BSA with 7% methanol and add 100 µl/well in rows B-C of the plate. Similarly add 100 µl diluted sample extract (1:10 in PBST-BSA) in rows D-E. Add 50 µl diluted AFB1-BSA-ALP conjugate (1:3000 in PBST-BSA) to all the wells and incubate at 37°C for 1 hour.

PBS-T wash

Substrate: Add pNPP prepared in 10% diethanolamine. Color develops in 20 minutes. Read the plate in ELISA reader at 405 nm.

AFB1-BSA; BSA block; Antibodies; Anti-rabbit-IgG-ALP; Aflatoxin; AFB_1-BSA-Enzyme.

Calculations: Take mean ELISA plate reading values (OD) for each standard and sample. Plot a standard curve by placing AFB1 standard concentration values on Y axis and respective OD values on X axis (Figure 2) on a semi-log graph paper. Correlate the unknown sample OD values where it touches the standard curve to determine the AFB concentration in the sample and determine the AFB content in the sample using this equation: AFB1 content in the sample (µg/kg) = (A*D*E)/G

A = AFB1 conc. (ng/ml) as determined from standard curve; D = Times sample dilution with buffer; E = Extraction solvent volume in ml (e.g. for 20 g sample 100 ml methanol); G = sample weight in grams.

Alternatively, use an Excel spreadsheet on the computer to draw a regression curve and using regression equation values, estimate aflatoxin in each sample. Additional information is available at www.icrisat.org/aflatoxin.

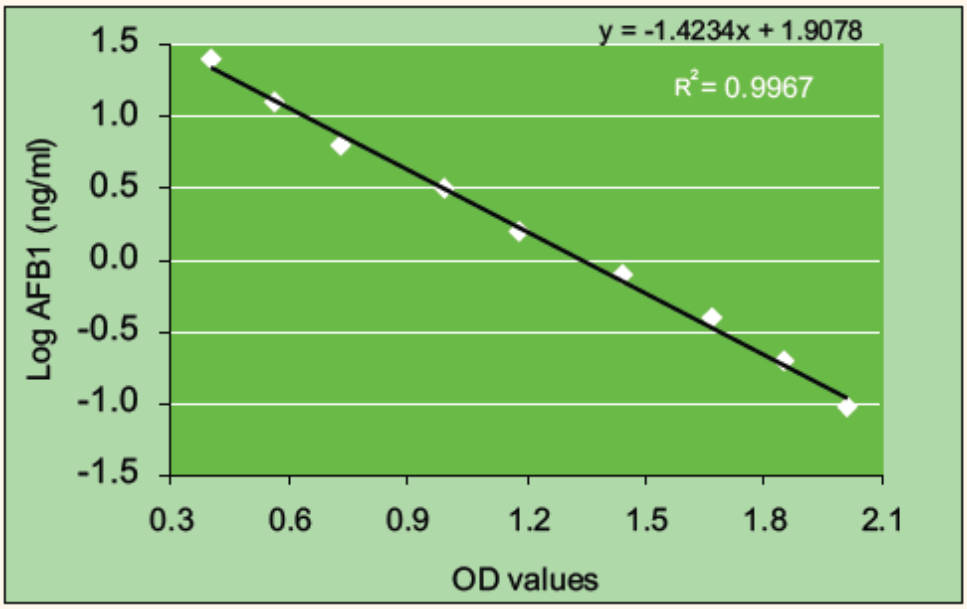


Figure 2: Regression/standard curve for aflatoxin estimation by ELISA.

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