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The evaluation of the most appropriate set of equipment and methods to be used by a laboratory responsible for the determination of aflatoxin in pistachio nuts.

SI/TUR/92/801

Technical report: Appropriate methods and equipment for an aflatoxin laboratory to monitor and study pistachio nuts.

Prepared for the United Nations Industrial Development Organisation,
acting as executing agency for the United Nations Development Programme

Based on the work of M. Nagler,
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United Nations Industrial Development Organisation
Vienna

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* This document is in first draft

EXPLANATORY NOTES

Glossary of Abbreviations and Terms.

Aflatoxin	A group of toxins mainly produced by the moulds *Aspergillus flavus* & *A. parasiticus* the most abundant & toxic of which is aflatoxin B_1. This is one of the most potent chemical carcinogens known as well as being an acute poison and an immune suppressant. Total aflatoxin is the sum of the quantities of aflatoxins B_1, B_2, G_1, and G_2.
AOAC	Association of Official Analytical Chemists
ELISA	Enzyme-linked immuno-sorbent assay. Advanced technology assay techniques available as packaged kits for aflatoxin testing.
Fluorimeter	An instrument for measuring the fluorescent intensity of solutions by a technique called fluorimetry. The equivalent American terms are fluorometer and fluorometry.
HPLC	High performance liquid chromatography. A high technology analytical technique for quantifying trace chemicals.
HPTLC	High performance thin layer chromatography. A high technology analytical technique capable of high sample throughputs.
Immuno-affinity methods	Utilise antibodies raised against aflatoxin which selectively capture aflatoxin molecules effecting a clean-up Aflatoxin is later released for detection or quantification. Marketed in kit form including ELISA.
IUPAC	International Union of Pure and Applied Chemistry
Mycotoxin	General term to describe toxic secondary mould metabolites which are toxic to humans and animals. Aflatoxin B_1 is one of over 300 known mycotoxins.

NRI	Natural Resources Institute. The UK Government Agency for providing overseas technical assistance
ppb	Parts per billion, or ug/kg. A unit of measurement of one part in 10^9.
ppm	Parts per million, or mg/kg. One part in 10^6.
Sub-sampling mill	Equipment designed to simultaneously comminute the sample and collect a representative sub-sample.
UNIDO	United Nations Industrial Development Organisation

ABSTRACT

This project aimed to identify, evaluate and recommend methods and associated equipment suitable for monitoring and studying levels of aflatoxin in pistachio nuts and kernels in a laboratory to be set-up by UNIDO in Turkey.

In the course of this project 5 types of mills and blenders were evaluated for sample preparation and it was found that a cutter / mixer mill with a 25 litre bowl was a very clear first choice. It was the only type of mill which could grind and prepare 10 kg primary samples of pistachio kernels and nuts in-shell. When fitted with a mixing attachment it could also produce a homogeneous water slurry from which an analytical sample could be drawn directly.

Three potentially fully quantitative aflatoxin assay techniques: TLC, fluorimetry and ELISA, were assessed. It was concluded that the laboratory should be equipped for both TLC and basic ELISA. TLC was selected because it could work down to the desired level of 4 ppb total aflatoxin, was relatively robust, low cost, suitable for small batches of samples, and was not dependent on importation of kits. The ELISA kit performed well, especially at the lower aflatoxin levels, and would be well suited for the assay of large numbers of samples at one time. It was rather complex to operate and uncertainties remained about how it would perform under Turkish conditions.

Four "rapid" aflatoxin assay kits were assessed for screening purposes and three of these were found to work to their specification with pistachio extracts. As these kits require little or no capital equipment it was recommended that a selection of two or three kits specified to work to 4 ppb total aflatoxin or 2 ppb / 5 ppb aflatoxin B_1 be purchased for further assessment and training purposes in Turkey.

A list of equipment, including safety items, was prepared.

TABLE OF CONTENTS.

INTRODUCTION.

This project was commissioned to select analytical methodologies and associated equipment to be employed in a laboratory which UNIDO plans to establish in Turkey to monitor aflatoxin in pistachio nuts. A consultant had previously visited Turkey, short-listed appropriate methodologies and produced a draft equipment list. The final choice of methodologies was complicated by the following factors:

a). A limited budget of $US 30,000

b). The need to work down to a 4 ppb total aflatoxin limit

c). Location of the laboratory in a private sector quality control laboratory.

The first choice techniques would normally be HPLC or HPTLC, but capital costs would be too high. Even if the budget was increased there would still be doubts about their suitability and sustainability in a quality control laboratory. It was therefore decided to identify a rapid, low-cost qualitative / semi-quantitative technique for screening batches of pistachio nuts down to the desired aflatoxin limit. This would be used in conjunction with a more quantitative, but relatively low capital cost, analytical and confirmatory method. Emphasis would also be placed on providing the laboratory with sample handling equipment which could retain the integrity of the primary sample.

The following Terms of Reference were specified:.

To perform and evaluate the following equipment and methods:

a). milling / sub-sampling equipment trials

b). Method evaluation, TLC and HPTLC

c). Method evaluation, rapid methods / kits.

d). Method evaluation, fluorimetry

e). Finalisation of equipment list.

I. PRELIMINARY SECTION

The laboratory needs to be able to monitor levels of aflatoxin down to 4 ppb total aflatoxin in pistachio nuts and kernels. This is the import limit set by the UK for pistachios ready for retail. A higher limit of 10 ppb total aflatoxin is allowed for nuts that are to be further processed. Germany and Spain also have low import limits: 5 ppb aflatoxin B_1 and 10 ppb total aflatoxin.

The methods have been evaluated and compared with consideration of the following criteria:

a). Ability to work to the desired aflatoxin limits

b). Ease of use

c). Time taken to obtain result

d). Robustness of method

e). Suitability for use in a quality control laboratory

f). Cost

g). Availability in Turkey (when known)

The following qualitative methods / kits were tested for their ability to work to a 4 and / or 10 ppb total aflatoxin limit:

Steiner TLC method (dilution to extinction mode)

Aflascan kit by Rhone-Poulenc

Biocode mini-column kit

EZ-Screen distributed by Rhone-Poulenc
Cite Probe by Idexx

Three methods were evaluated for their suitability as fully quantitative assays, these were:

Steiner TLC method (comparison with standards mode)

Fluorimetry (following immuno-affinity clean-up)

Biokits Total Aflatoxin ELISA kit by Cortecs

Fluorimetry is being considered because, with the advent of highly specific immuno-affinity clean-up columns, there has been renewed interest in this technique as a means of quantification. An AOAC / IUPAC collaborative study (2) has been conducted to validate a fluorometric method for determining total aflatoxin in maize, groundnuts, and peanut butter. As a result the method has been adopted official first action by the AOAC. A broadly similar method has been devised by Biocode (4) for use with maize.

ELISA is being evaluated because capital expense is relatively low: microtitre plate readers cost from about US$ 4000, and it is a proven quantitative technique.

II. EXPERIMENTAL

IIA. Milling / Sub-Sampling Mill Trials.

Sub-samples (500 g to 2.5 kg) of pistachio kernels and pistachio nuts in-shell to be used to form an assessment of the suitability and efficiency of the following mills:

Swift / NRI Sub-sampling mill
Apex Knife mill
Cutter / Mixer mill, 25 l capacity (Hobart VCM-40, discontinued, now Stephan UM 25)
Hobart Vegetable Slicer Mk. II.
Waring 4 litre blender

i. Swift / NRI Sub-sampling mill

A sub-sampling mill manufactured by Swift Engineering Ltd, UK was available for evaluation. This mill, which had been developed in collaboration with NRI, comminutes the sample, forces it through a screen and then collects two representative 5% sub-samples. Previous trials with the mill had shown it to work satisfactorily with corn and groundnuts.

Five hundred gramme sub-batches of pistachio nuts in shell and pistachio kernels were fed through the mill fitted with a 3 mm diameter screen and operating at slow (4 mA), medium (6 mA) and high (8 mA) motor speeds. The vibrator feed was set to the slowest rate, and then gradually increased until the motor started to slow significantly and the load increase above 9 Amp (the cut-out was set at a 10 Amp load).

ii. Knife mill.

An Apex 20 mm diameter knife mill with a range of screen options ranging from 1 mm to 8 mm was evaluated. This is the mill used by NRI for most of it's routine mycotoxin samples.

Five hundred gramme sub-samples were fed into the mill using a hopper with manual feed-rate control

iii. Cutter / Mixer mill, 25 l capacity (Hobart).

A Hobart cutter / mixer fitted with a cutter blade and operated with manual mixing was evaluated. It could take up to a 10 kg sample and after processing it could easily be emptied by use of a tilt mechanism. Water slurries could also be produced directly in the mixer, after sample division. The cutter / mixer tested is no longer marketed by Hobart, but it is still available from Stephan Machinery UK Ltd., the manufacturers, as model UM 25.

IIB. Method Evaluation.

The following methods: TLC (Section IIIB), kits (IIIC), and fluorimetry (IIID) were assessed to establish their suitability for working to 4 ppb and 10 ppb total aflatoxin levels in pistachio kernels and pistachio nuts in-shell.

Extracts of pistachio were spiked with aflatoxin at levels of 4, 10, and 20 ppb total aflatoxin and these contained 1.6, 4.1, and 8.1 ppb aflatoxin B_1 respectively. The spiked extracts were prepared by adding the calculated volume of standard in benzene : acetonitrile to a vial using a Hamilton syringe. The standard was taken down to dryness on a sample concentrator over nitrogen at 45 ^{o}C and then transferred quantitatively to a volumetric flask using aflatoxin-free sample extract. The volumetric flask was then filled to the calibration mark with sample extract.

i. Thin Layer Chromatography (TLC)

The method described by Steiner (1) was followed. This is outlined in the Results and Discussion Section where performance of each step is assessed.

ii. Immuno-affinity and ELISA kits.

Each kit came with full instructions and these were followed as closely as possible with the following exceptions:

Biocode Mini-column. The extraction procedure stated was not consistent with well known and widely accepted criteria. Hence the meal to solvent ratio was increased from 1:2 to 1:5 and volumes used in later steps were amended to retain a 1 g sample weight on the affinity column.

Aflascan kits. In addition to use of the syringe for forcing liquids through the immuno-affinity column, an over-pressure compressed air system was used to ease the operation and allow up to 8 simultaneous assays.

ELISA kit

The Cortecs kit comes complete with aflatoxin standards and all reagents, save extraction solvent. The procedure is quite complex and it is easy to make a mistake. To minimise this risk a bench-overlay was designed and produced. An outline of the method is given in Annex 3. A Dynatech Microelisa Minireader MR590 manual plate reader was used to read the plate.

The kit is designed to be used under ambient room temperatures in the range 19 to 23 ^{o}C.

iii. Fluorimetry.

Evaluation of this methodology was carried out using a fluorimeter loaned by Jenway (Model 6200) fitted with a 360 nm incidence filter and a 425 nm band-pass filter on emission. The specification claimed detection down to 1 ng / litre quinine sulphate in 0.1 M sulphuric acid, which should equate to around 0.6 ppb total aflatoxin.

III. RESULTS AND DISCUSSION

IIIA. Milling / Sub-Sampling Mill Trials / Sample Preparation.

i. Sub-sampling mill.

Pistachio kernels: Initially a medium motor speed and slow vibrator feed was used (about 1 kg / 17 min.). As the vibrator speed was gradually increased to about 1 kg / 10 minutes the motor slowed dramatically and then the motor cut out. The screen was found to be almost completely blocked with oily pistachio paste and there were large balls of paste inside the screen which must have been slowing the cutter blades which in turn would have caused the over-load cut out.

After cleaning and resetting, a fast motor speed was used in conjunction with a slow feed rate of 1 kg / 17 minutes. Again only the slowest feed rates could be used. After the 500 g of kernels had been milled the screen was found to be partially blocked over the lower third portion, but there was no build-up of paste inside the screen to seriously slow the motor. It was estimated that screen would require cleaning after milling each 1 kg., but there were insufficient kernels left to check this estimate.

Nuts in shell: A fast motor speed and slow feed rate was used initially. As the feed rate increased the motor speed slowed and the load increased alarmingly. The mill was quite noisy to operate with nuts in shell and small particles of nuts escaped from the top of the mill. After the trial the screen was found to be completely clean, with no sign of the paste found when milling pistachio kernels. This trial was repeated with a second batch of 500 g of nuts. Again it was not possible to increase the feed rate significantly, even using the fastest motor speeds attainable. The milling took 8.5 minutes to complete which means that it would take 170 minutes to mill a 10 kg sample.

The trial was repeated using a slow motor speed, but again it was not possible to increase the feed rate without threatening a motor cut out. The screen was found to be partially blocked with paste and large quantities of unmilled whole nuts and shells were found within the screen.

The sub-sampling mill was found to be unsuitable for use with either pistachio kernels or pistachio nuts in shell due to low throughputs and, in the case of kernels, blocked screens.

ii. Knife mill.

Pistachio kernels: The mill produced a paste which quickly blocked the available screens. The mill could only be used if the kernels were mixed 50:50 with a suitable inert compound, such as oyster shell (3, Section 974.16), or if milled in shell.

Nuts in Shell: Using a 5 mm screen the product was reasonably free-flowing and was considered suitable for sample division. However, the milling rate to produce this was still very slow at about 1 kg in 10 minutes. Faster milling was obtained using an 8 mm screen but particle size was considered too large.

The knife mill would not be suitable for producing free-flowing meal from nuts in shell (too slow) or from kernels. It could be used for course milling nuts in shell prior to sample division and slurry preparation, but it would only work with pistachio kernels if 50:50 oyster shells were added (although this would tend to blunt the knives).

iii. Cutter / mixer mill (25 l).

Pistachio kernels: The mill, fitted with a cutter blade, produced a moderately fine, fairly free-flowing product in a very short time: 2.5 kg took 5 minutes. It was found that feeding the kernels from the top with the mill running resulted in a much more consistent particle size than that obtained when the bowl was filled prior to milling. The ground material was suitable for sample division and slurry preparation. The latter operation could be carried out directly in the Hobart mixer, see Slurry section below.

Nuts in shell: The mill, fitted with a cutter blade, produced a mix of predominantly large-particle size shell and well milled, free flowing, kernel in a very short time (2.5 kg in 5 minutes). The product was sieved through a 4.75 mm screen and the milled product was found to constitute 63 % by weight. Hence shells, plus a very few kernels, representing 37% by weight could be removed prior to sample division and slurry preparation.

The Hobart mixer was found to be the most suitable type of equipment for milling primary samples of both pistachio kernels and pistachio's in shell and should be included on the equipment list.

iv. Sample preparation: slurry production

a). In a 4-litre Waring blender.

The relatively large particle size produced by a knife mill fitted with a course screen, or by a Hobart Cutter / Mixer mill could result in loss of sample integrity and lead to a high variance associated with subsequent sample division. To reduce this problem, and assist with the extraction step, it would be advisable to reduce the sample to no less than 1 kg

and then homogenise with water in a blender or emulsifier to produce a water slurry.

The slurry procedure was carried out using a Waring blender and a variety of meal:water ratios. It was found that an excellent slurry could be produced with kernels using a ratio of meal : water of 1 : 2. A good slurry could also be obtained from course-ground nuts in shell using a ratio of 1 : 3.

b). In a cutter / mixer mill.

It was found that the best slurry could be produced by fitting the mill with a mixing blade and loading 4 litres of water and 3 kg of course-ground pistachio kernels. A good slurry was produced after 5 minutes of mixing.

The mill was best cleaned by use of a water hose, and this should be considered when selecting a location within the laboratory.

Slurries should always be used when analysing pistachio kernels, and would offer advantages when analysing whole nuts. A Waring 4 l blender was suitable for samples weighing up to 1 kg, and the Hobart Cutter / Mixer was suitable for sample weighing up to 5 kg.

IIIB. Method Evaluation, TLC / HPTLC.

The Steiner *et al* (1) metnod was assessed using samples of pistachio kernels and pistachio's in-shell each spiked at 0, 4 and 10 ppb total aflatoxin.

Following extraction with aqueous methanol, 70 ml aliquots of filtrate, each representing 20 g of sample, were spiked with aflatoxin at the three levels. The hexane wash removed a green pigment, leaving the aqueous solution orange in colour.

Partition of aflatoxin into dichloromethane removed most of the colour, although when evaporated to dryness there was a significant residue of impurities. This was particularly apparent for the extracts obtained from nuts in-shell.

Thin layer chromatography was carried out using both silica TLC plates (Merck No. 5553) for semi-quantitative visual assessment and HPTLC plates (Merck No. 5547) for fully quantitative determination by fluorodensitometer to obtain recovery data.

A TLC plate was spotted with 5 and 10 Ìl spots of each extract and standards were spotted at 1, 2, and 3 Ìl volumes using microcaps and a semi-automatic spotter (CAMAG Nanomat III). Following development, the TLC plate was found to be free of interfering spots or streaks, although the spots were fairly diffuse. Use of benzene instead of toluene in the spotting solvent would make the spots more compact, but would introduce a safety hazard.

It was found that 5 Ìl spots were just visible at the 10 ppb total aflatoxin level (B_1 = 4.4 ppb), but could not be detected at the 4 ppb total aflatoxin level. Aflatoxin at 4 ppb total aflatoxin could JUST be detected in the 10 ul spots, but this is subjective and will depend on the keenness of the analysts eye-sight.

Hence a very simple quality control procedure can be implemented, based on the Steiner TLC method. The method will take around 90 minutes to complete for a single sample, but it should prove possible to analyse 4 samples in 2 hours.

HPTLC showed that recoveries for the analytical method were good. For pistachio kernels recoveries at 4 and 10 ppb total aflatoxin were 75 and 88% respectively and for nuts in-shell the recoveries were 66% and 81%.

With suitably dilute standards it would be possible to quantify using comparison with standards. It was found that HPTLC grade plates (Merck 5547) gave more compact aflatoxin spots after development and were therefore preferred for quantification by comparison with standards.

The Steiner analytical method was found to be an excellent choice for aflatoxin quality control of pistachio nuts.

IIIC. Method Evaluation, Kits.

The following kits were evaluated:

Aflascan by Rhone-poulenc

Biocode Mini-column

Cite Probe by Idexx UK.

EZ- Screen, Quik-Card Test by Environmental Diagnostics Inc..

Biokits Total Aflatoxin Assay by Cortecs Diagnostics Ltd.

The first four kits listed work to pre-determined aflatoxin limits and they are possible alternatives or complements to the Steiner TLC method. The fifth kit is designed to be fully quantitative, and it's suitability needs to be compared with that of the fluorometric method, which is evaluated in Section IIID, and the TLC method using comparison with standards which is evaluated in Section IIIB.

All immuno-assay kits must be stored at 2 to 8 ^{o}C and used before the expiry date.

i. **Aflascan**

Manufactured by Rhone-Poulenc Diagnostics Ltd., Montrose House, 187 George Street, Glasgow G1 1YT, UK.

Cost: In UK, 180 + VAT for 25 tests, or 7.20 (US$ 10.80) per test, excluding extraction solvent and filter paper costs.

The kit comprises: a hand pump unit (plastic syringe), a glass "syringe barrel", an immuno-affinity column, a florisil tip for trapping aflatoxin and a colour comparator for reading aflatoxin ranges according to the fluorescent intensity of a blue band in the tip when viewed under long-wave ultra-violet light.

The kit methodology can be adapted to work to 10, 5, 4, or 2 ppb total aflatoxin limits as the volume of extract applied to the column is increased from 10 ml through 20, 25 to 50 ml for detection of 2 ppb. In this study 25 ml of extract was added to check the zero and 4 ppb spikes and 10 ml of extract was used for the 10 and 20 ppb spikes.

The maximum flow rate allowed was 3 ml per minute, so this required application of steady pressure on the hand pump for about 9 minutes to pass 25 ml of extract. Washing the column with 2 x 10 ml water takes another 7 minutes of steady exertion on the hand pump. Whilst this was acceptable for occasional use, the novelty soon wore off and an over-pressure system was used instead. This enabled up to 10 Aflascan assays to be performed simultaneously and would be essential if Aflascan was selected as a method of choice for the laboratory.

Time for 1 assay: 35 minutes.
Time for 6 assays: 70 minutes using over-pressure system

Ease of use: Generally straightforward, but partition of aflatoxin into chloroform and application of the lower chloroform layer onto the florisil tip was a little tedious and required some practice. The "comparator" worked quite well, but the blue band was not always spread evenly around the florisil tip.

Results:

Spike (ppb)	Comparator Reading	Correct	Comment
Kernels:			
0	0	Yes	
4	4	Yes	Slightly less
10	10	Yes	Slightly less
20	20	Yes	>>10, but <20
Nuts in-shell:			
0	0	Yes	
4	4	Yes	Compact, close
10	10	Yes	Clear, but <10
20	20	Yes	>>10, but <20

Table 1. Results obtained using the Aflascan kit on spiked pistachio extracts.

Readings using the comparator gave the correct result at all 4 levels for both pistachio kernels and pistachio nuts in-shell. The reading was taken as the closest comparison of blue intensity, even if the intensities were not exactly matched. In practice any clearly visible blue fluorescence would be interpreted as being at, or above, the appropriate detection limit and 4 ppb total aflatoxin could be clearly detected.

Special Equipment Required:

UV viewing cabinet fitted with 366 nm light.

Air compressor and over-pressure rig highly desirable.

Assessment: Aflascan gave good performance at both 4 ppb and 10 ppb total aflatoxin limits. Not as fast or simple to perform as some tests, but would be worth considering if supply and cost factors were favourable in Turkey.

ii. Biocode mini-column

Manufactured by Biocode Ltd., University Road, Heslington, York Y01 5DE.

Cost:	Box of 10 Easi-Extract columns	58
	Box of 10 semi-quantitative analytical columns	28
	Standard columns	125

Cost per assay: 8.60 + 1 off 125 for standards.

This assay requires the use of an Easi-Extract immuno-affinity column clean-up followed by partition into chloroform before a conventional mini-column clean-up with visualisation on a florisil layer under long-wave UV light. The intensity of the fluorescent band is then compared to that of standards.

Extracts of pistachio kernels and nuts in shell were spiked to 0, 4, 10, and 20 ppb total aflatoxin and the procedure was followed.

Results:

The resultant mini-columns were opaque relative to the standard columns, but this effect was minimised by blowing the columns dry with nitrogen.

A yellowish interfering band was observed on the florisil interface for all samples.

Spiking level	Comparison	Correct
Kernels:		
0	0	Yes
4	0	No
10	4	No
20	10	No
Nuts In-Shell		
0	0	Yes
4	0	No
10	4	No
20	10	No

Table 2. Results obtained using the Biocode Mini-column kit on spiked pistachio extracts.

The Easi-extract mini-column assay appeared to suffer matrix effects and did not give correct results. This was a surprising finding because on paper it appeared to be a more rigorous method than Aflascan.

Time for 1 assay: 40 minutes
Time for 6 assays: 75 minutes

Ease of use: Similar to, but somewhat less easy to use than Aflascan. Additional column washing with buffer and dilution of sample extracts with buffer was required. The mini-columns took longer to run than the florisil tips

Special Equipment Required:

UV viewing cabinet fitted with 366 nm light.
Air compressor and over-pressure rig essential.

Assessment: **This trial indicated that the Biocode mini-column may not be suitable for screening pistachio kernels and nuts in-shell.**

iii. Cite Probe.

Manufactured by Idexx UK., The Old Court House, Hughenden Road, High Wycombe, Buckinghamshire, HP13 5DT.

Cost: 3.75 each.

This was the neatest looking kit reviewed. It consisted of a plastic moulding containing 4 wells, a plastic probe complete with filter, and a dropping pipette. All reagents were already present in the wells and in the probe. The kit was designed to detect 5 ppb aflatoxin B_1.

Time for 1 assay: 3 minutes
Time for 6 assays: 18 minutes

Ease of use: Operation could hardly be easier. Eight drops of extract are added to the first well and the probe is firmly inserted into the well for 2 minutes. The probe is then placed into the second well and the green button on top of the probe is firmly depressed. After thirty seconds the probe is placed in the third and fourth wells sequentially for 30 and 15 seconds respectively. The probe is then inverted and the colour of a circular control port is compared with that of the sample port. The control port should go a dark blue colour to show that the kit is working properly. If the sample also goes dark blue, then the sample contains less than 5 ppb aflatoxin B_1, but if the port stays a lighter colour than the control then the sample is adjudged to contain more than the limit

Results:

Spike, Total Aflatoxin ppb	Aflatoxin B_1 ppb	Reading	Correct
Pistachio Nut:			
0	0	-'ve	Yes
4	1.6	-'ve	Yes
10	4.1	-'ve	Yes
10 (Repeat)	4.1	+'ve	No
20	8.1	+'ve	Yes
Pistachio kernel:			
0	0	-'ve	Yes
4	1.6	-'ve	Yes
10	4.1	-'ve	Yes
10 (Repeat)	4.1	+'ve	No
20	8.1	+'ve	Yes

Table 3. Results obtained using the Cite Probe kit on spiked pistachio extracts.

The kit worked well to a 5 ppb aflatoxin B_1 limit with the 4.1 ppb spike sometimes positive and sometimes negative, as would be expected. The results suggest that no serious matrix interference exists when the kit is used for testing pistachio nuts and kernels and the kit may be suitable for screening batches at the 5 ppb aflatoxin B_1 limit or when working to a 10 ppb total aflatoxin limit. It could not, however, work to the 4 ppb total / 2 ppb aflatoxin B_1 limit and this will limit it's applicability.

Special equipment required: None, but a special probe reader is available from the manufacturers to make the test objective.

Assessment: Cite Probe was extremely easy and quick to use, and it appears to give reliable results with pistachio samples when working to a 5 ppb aflatoxin B_1 limit, but it

has limitations when working down to a 4 ppb total aflatoxin limit.

iv. EZ-Screen Quik-Card Test (5 ppb)

Manufactured by Environmental Diagnostics, Inc., 1238 Anthony Road, Burlington, NC 27215, USA. and distributed by Rhone-Poulenc.

Cost: 135 for 25 x 6 site cards (5 ppb)
1.08 per sample

This kit was not tested as comprehensively as the others. A single 6 spot card was available and this was used to test extracts of nuts in-shell spiked 0, 1.6, 4, and 8 ppb total aflatoxin. The card can not detect aflatoxin G_2, which is in fact rarely found in significant quantities in nature, so the spiking levels were effectively 0, 1.3, 3.2, and 6.3 ppb aflatoxins $B_1+B_2+G_1$.

Extraction is with 80% methanol-water, but a meal to solvent ratio of only 2 is used and this will not give optimal extraction of aflatoxin from the commodity.

All reagents were provided in droppers containing glass ampoules, and these needed to be carefully crushed immediately prior to use. A Negative Control was provided.

The procedure involved sequentially spotting diluted sample, enzyme conjugate, wash, and a substrate reagent to develop colour in each port after a 5 minute development time.

Time for 1 card (up to 5 samples): 7 minutes.

Ease of use: Much easier to use than the kits employing affinity column clean-up, but not quite as simple as the Cite Probe.

Results:

Spike, Total Aflatoxin B_1	Result	Correct
Control	Dark colour	Yes
0	-'ve	Yes
1.6	-'ve	Yes
4	+'ve	No
8	+'ve	Yes

Table 4. Results obtained using the EZ-Screen Quik Test kit on spiked pistachio extracts.

Special Equipment Needed: None

Assessment: This first look at the EZ-Screen indicated that it may prove applicable for screening pistachio nuts and kernels working to a 5 ppb aflatoxin B_1 + B_2 + G_1 limit. It is relatively inexpensive, and can be photocopied for archiving. The kit is worth a more detailed assessment in Turkey, cost and availability permitting.

v. Biokits Total Aflatoxin Assay Kit.

Manufactured by Cortecs Diagnostics Ltd., Tech Base 1, Newtech Square, Deeside Industrial Park, Deeside, Clwyd, CH5 2NT, UK.

Cost 230 +VAT for 24 samples (3 session) to 37 samples (1 session) to give a cost per sample of between 9.58 down to 6.21 if all 37 samples are ready for quantification at the same time.

This is a competitive ELISA kit which contains a microtitre plate and all standards and reagents required, although some of these need diluting prior to use. The procedure is complex and involves pipetting sequentially small volumes *very accurately* into up to 96 wells. Great care and concentration is needed to ensure that the correct volume of

the correct reagent is pipetted into the correct well at the correct time. A desk overlay was produced to help minimise mistakes. A quiet room with temperatures in the range 19 to 23 ^{o}C is preferred. If temperatures exceed 23 ^{o}C, then it is possible to shorten incubation times to compensate, but the technique becomes less robust.

The procedure is outlined in Annex 2.

The following times to complete the assay do not include sample preparation and extraction and assume manual pipetting

Time for 8 assays: 4.5 hours (33 minutes per sample)
Time for 16 assays: 5.0 hours (19 minutes per sample)
Time for 37 assays 6.0 hours (10 minutes per sample)

Ease of use: This kit was not particularly easy to use, but given training and good equipment it was not too daunting. It must be remembered that alternative quantitative techniques, such as HPLC and HPTLC require additional clean-up steps and time per sample is actually much longer (HPLC takes at least 25 minutes per sample just for the quantification step).

Special Equipment Required: High precision direct displacement pipettes for dispensing 50 and 100 ìL, and an air replacement pipette for dispensing 2,400 ìl are required. A plate shaker and a microtitre plate reader are also essential. The latter can range in price from manual instruments costing around US$ 4,500 to computer controlled plate readers costing US$ 15,000 or more. An air-conditioned room with temperature control in the range 19 to 23 ^{o}C is also highly desirable, but not absolutely essential. At higher room temperatures the incubation times can be reduced, but trial and error will be required rather than standard operating procedures.

The kit was tested by spiking extract of pistachio kernels and extracts of pistachio nuts with 0, 4, 10, 20, 50, and 200 ppb total aflatoxin. In addition extracts of the 10 kg kernels (PK2) and nuts in-shell (PN2) supplied for milling trials were quantified.

A calibration curve was drawn, see Annex 3, and the following results were obtained.

Results:

Sample	Absorbance			Aflatoxin
	1	2	Mean	Content ppb
Kernels:				
0	1.99	2.00	1.995	-'ve
4	1.42	1.57	1.495	5.6
10	1.31	1.32	1.315	8.7
20	0.96	0.91	0.935	18.0
50	0.64	0.62	0.63	34.0
200	0.33	0.33	0.33	130
Nuts:				
0	1.87	1.96	1.915	-'ve
4	1.49	1.49	1.49	5.7
10	1.18	1.12	1.15	12.0
20	0.72	0.77	0.745	26.0
50	0.42	0.43	0.425	72.0
200	Error			
PK2	1.94	1.89	1.915	-'ve
PN2	1.99	1.98	1.985	-'ve

Table 5. Results obtained using the Biokits Total Aflatoxin ELISA kit on spiked pistachio extracts.

The kits could easily detect and quantify at the 4ppb and 10 ppb total aflatoxin levels, and in fact results for these low levels were more reliable than for the high ones, where a slight uncertainty concerning the best calibration curve

could cause significant differences in results. It was noted that the kernels tended to give lower results than the nuts.

It is interesting to note that the 10 kg samples of kernels and nuts in-shell both gave negative aflatoxin results.

Assessment: Biokits ELISA kit could provide the required quantitative data and had sufficiently low detection limits to work at and below the 4 ppb total aflatoxin level. Questions remain as to the performance of these kits under the conditions prevailing in the GTS quality control laboratory in Gaziantep: there may also be seasonal effects. Provided that a well and reliably air-conditioned laboratory area can be allocated, it is recommended that the modest outlay required on capital equipment and trial kits be made to allow further assessment. A deciding factor is the possibility of also using the ELISA equipment for pesticide assays: a number of kits are available.

IIID. Method Evaluation, Fluorimetry.

With the advent of highly specific immuno-affinity clean-up columns there has been renewed interest in fluorimetry as a means of quantification. An AOAC / IUPAC collaborative study (2) has been conducted to validate a fluorometric method for determining total aflatoxin in maize, groundnuts, and peanut butter. As a result the method has been adopted official first action by the AOAC. A broadly similar method has been devised by Biocode (4) for use with maize.

These methods were evaluated using a fluorimeter loaned by Jenway (Model 6200) fitted with a 360 nm incidence filter and a 425 nm band-pass filter on emission. The specification claimed detection down to 1 ng / litre quinine sulphate in 0.1 M sulphuric acid, which should equate to around 0.6 ppb total aflatoxin.

Calibration was attempted using both quinine sulphate at 34 ng / litre (equivalent to 20 ppb) and with spiked methanol standards at 0, 10, 20, 50, and 100 ppb total aflatoxin. The fluorescence enhancer recommended by Biocode, 0.05 mg / litre pyridinium bromide perbromide, was used.

The quinine sulphate standard gave a steady reading, but this was not consistent with the freshly prepared methanolic standards. Results for the methanolic standards were unsteady and inconsistent and the fluorescent enhancer did not appear to work. Readings fluctuated wildly when degassing occurred, so the cuvette was tapped gently to release the air bubbles. At one stage it was noticed that the 1 ml of methanol and 2 ml water were not completely miscible, so mixing was increased. We checked the filters supplied, and found them to specification. We sought technical advice from Biocode and Jenway.

However none of these procedures, conducted over a four day period, could improve the performance of the method in our hands.

We spiked extracts of pistachio kernels and pistachio nuts in-shell at 0, 4, 10 and 20 ppb total aflatoxin and carried out clean-up using both Rhone-Poulenc Aflaprep and Biocode Easi-Extract plus alumina column procedures. We checked to see if the resultant extracts were consistent by calibrating on the 20 ppb sample and seeing if the other extracts were consistent. They were not. In one case the unspiked sample fluoresced more brightly than the 20 ppb sample (according to the fluorimeter).

We are at a loss to explain the poor performance of fluorimetry, when it appears to have worked so well in the 11 laboratories collaborating in the AOAC/IUPAC study.

Assessment: It is not possible to recommend the use of fluorimetry on the basis of our evaluation, but if the system can be successfully demonstrated at a later date, preferably in Turkey, the decision could be reconsidered for any future application.

IIIE. Comparative Assessment of Analytical Techniques.

i. Qualitative methods working to an aflatoxin limit.

The TLC method and the Aflascan kit could both work to a 4 ppb and 10 ppb total aflatoxin limit. The kit was easier to perform, required far less laboratory equipment and a result was obtained in about 35 minutes compared to 90 minutes by TLC. The main disadvantages of relying on a kit are possible uncertainties concerning the supply of the imported item, and the quality on receipt bearing in mind that ideally the kit should be stored at 2 to 8 ^{o}C.

TLC had the advantages that a positive sample could easily be quantified and confirmed and that running costs were significantly lower at around 1.50 in consumable costs.

It was concluded that TLC method should be installed as the basic method, but that it could be complemented by kit methods.

Although the EZ-Screen works to 5 ppb aflatoxin B_1 + B_2 + G_1 (no G_2) instead of 4 ppb it will often identify samples at, the desired limit and should identify samples just above the limit. Further evaluation in Turkey will assess it's usefulness for aflatoxin screening in the market chain. Certainly, it was easy to perform and was the cheapest and the second quickest of the kits.

The only disadvantage of the Cite probe is the 5 ppb aflatoxin B_1 limit which limits it's application. A positive probe would certainly identify samples exceeding a 4 ppb

total aflatoxin and would question samples needing to satisfy a 10 ppb total aflatoxin limit. Samples containing less than 5 ppb aflatoxin B_1, but more than 4 ppb total aflatoxin might test incorrectly. The Cite probe might be ideal for monitoring any exports to Japan, which has a 5 ppb aflatoxin B_1 limit. This probe could well prove popular for application in the marketing chain because it is the easiest kit to use.

Screening batches to detect those containing more than a prescribed limit of aflatoxin can never be an exact procedure, even using the most rigorous sampling and sample handling techniques and the most accurate and precise assay method. There will always remain a considerable degree of uncertainty concerning batches containing aflatoxin at or close to the limit. Bearing this in mind, the performance of the kits over a 1 or 2 ppb range close to the limit may not be a limiting factor.

Criteria	Biocode	EZ-S	Cite	Aflascan	TLC
Limit ppb	4,10	5T-1	$5B_1$	4,10	4,10
Time/assay	40	7	3	35	90
Time/6 assay	75	7*	18	70	120
Ease (1-5) 5=easiest	3	5	5	4	2
Cost/assay	8.60	1.08	3.75	7.20	1.50
Correct (1-5) 5=all correct	1	5	4	5	5
Extra Eqt.	Yes	No	NO	No/Yes	Yes

Table 6. Comparative assessment of qualitative assays

* 5 samples only ie. one card.

Times are in minutes and cost is in sterling.

Key:

Extra Eqt. = Need for equipment additional to that provided with the kit.

Correct = Was the correct result obtained?. Scaled from 1 (bad) to 5 (all correct)

T-1 = Total aflatoxin minus aflatoxin G_2.

ii. **Fully quantitative methods.**

The three methods evaluated were: TLC by comparison with standards, Biocode Total Aflatoxin ELISA kit by Cortecs, and fluorimetry.

Unfortunately, and for reasons unknown, the fluorimetry method did not work in our hands. It has been shown in a collaborative study to perform well, so perhaps it is harsh to discount it completely on the basis of this one trial. However, both the TLC and ELISA performed well and these can be compared.

If the supply of ELISA kits in good condition could be assured, then this might be the method of choice. No clean-up procedure is required and up to 37 samples can be quantified in duplicate by ELISA in about 5 hours. Using TLC it would be difficult to analyse 12 samples in the same time.

The ELISA kit had excellent quality assurance built into the procedure so it would be easy to detect if anything was going wrong. The problem is that there is a lot to go wrong, so one fears that plates might need to be repeated. This could work out very expensive and extremely frustrating. Only further trials, in-country, can demonstrate the reliability of the method.

It is therefore recommended that TLC be the main method of quantification but that basic ELISA equipment be installed for further evaluation of this technique.

IV. CONCLUSIONS.

1. A cutter / mixer mill with a 25 litre bowl, see the equipment list for a full specification, was found to be the equipment of choice for milling 10 kg primary samples and preparing analytical samples of both pistachio kernels and pistachio nuts in-shell. (We used a Stephan UM-25 cutter / mixer mill).

2. The TLC method evaluated (1) was found to be the method of choice for working to a 4 and 10 ppb total aflatoxin limit in a qualitative mode. Although it took longer to perform than the immuno-assay kits, it was cheaper to operate and should certainly be available because it is not dependent on imports of sophisticated kits which may have supply or quality problems from time to time.

3. Aflascan, EZ-Screen and Cite Probe immuno-assay kits were found to perform satisfactorily when working to 4 and / or 10 ppb total aflatoxin limit and of those tested only the Biocode mini-column kit appeared to suffer from matrix interference.

4. The TLC method was shown by HPTLC to give good recoveries (70 to 80%) and could be used to provide semi-quantitative data by a subjective comparison with standards technique. The method also allows confirmation by hemi-acetal derivatisation using tri-fluoroacetic acid.

5. Objective quantification down to the required aflatoxin limits was attained using a Biocode ELISA kit, but the suitability of this kit for operation in Gaziantep needs to be established.

6. Fluorometric methods cannot be endorsed on the basis of our studies, but neither can they be discounted because they do appear to work in other laboratories.

V. RECOMMENDATIONS

1. The aflatoxin laboratory should adopt the Steiner (1) method for determination of total aflatoxin in pistachio kernels and pistachio nuts in-shell, and the laboratory should be suitably equipped.

2. A cutter / mixer mill with a 25 l bowl should be purchased for milling primary samples and preparing homogeneous slurries.

3. The laboratory should be equipped for carrying out quantitative aflatoxin analysis by ELISA and kits such as Biokits by Cortecs should be further evaluated. The equipment would also be suitable for carrying out other ELISA tests, such as those designed to determine selected pesticide residues.

4. Rapid, simple, immuno-assay aflatoxin field tests kits, such as EZ-Screen and Cite Probe, should be purchased for training and for further evaluation of suitability for use in pistachio aflatoxin quality control at the trader level.

5. Aflascan kits and EZ-Screen tests should be evaluated in Turkey as possible laboratory based screening methods to complement TLC.

VI. APPENDIX.

ANNEX 1.

Equipment List.

ALL ELECTRICAL EQUIPMENT MUST BE OF THE CORRECT VOLTAGE AND FREQUENCY FOR OPERATION AT THE GTS LABORATORY, GAZIANTEP, TURKEY (CHECK THAT 220 V, 50 Hz IS CORRECT)

A. Equipment for milling, sample preparation and extraction.

Item No.		Description	Cost (US$ '000)
1.	1	Cutter / mixer mill, 25 litre, stainless steel bowl, speed 1500 /3000 rpm, electronic brake, manual mixing baffle, tilt mechanism for unloading and cleaning, eg. Stephan UM-25, see Annex 2.	4.5*
2.	1	Cutter blade for cutter mixer	0.3
3.	1	Stirrer blade for cutter mixer	0.3
4.	1	Mixer blade for cutter mixer	0.3
5.	1	Blender, 4 litre, high speed, with stainless steel container eg. Waring. (For slurry prep.)	2.7
6.	1	Spare blender blades, blending assembly for SS910, & blender coupling assembly for CB6 base unit, and lock nut wrench	0.5
7.	2	Blender, 1 litre, heavy duty, with 1 litre stainless steel container (Explosion proof not required)	1.3
8.	4	Stainless steel 1 litre containers for blender	0.7
9.	1	Spare blades and seals for steel containers	0.2
10.	10	Filter funnels, 15 cm diameter, glass.	0.15
11.	10	Filter paper, Whatman No. 2, 32 cm.	0.30

12.	20	Flasks, 500 ml; pyrex, graduated with plastic screw cap with PTFE liner	0.27
13.	10	Flasks, 250 ml, pyrex, graduated with plastic screw cap with PTFE liner	0.12
14.	10	Spare caps for flasks	0.03
15.	10	Measuring cylinders, 250 ml, grad. SBW borosilicate glass.	0.07
16.	10	Measuring cylinders, 100 ml, grad. SBW borosilicate glass	0.05
17.	5	Measuring cylinders, 10 ml, grad. SBW borosilicate glass	0.04
18.	1	Balance, top pan to 5 kg for weighing analytical samples and reagents, eg. Sartorius	1.5
19.	1	Balance, to 20 kg for weighing incoming samples.	0.2
B.	Equipment for TLC method.		
20.	10	Separating Funnel, 500 ml pear shaped with glass stopper and stopcock	0.50
21.	1	Rotary evaporator with temp. control water bath. Standard model eg. Bibby	1.5
22.	1	Vacuum pump, 20 inches Hg (20 Torr). Diaphragm type suitable for rotary evaporator, 5 m^3 / hr, 3 x 10^{-2} mbar.	1.4
23.	1	Cold trap for above vacuum pump	0.04
24.	2	TLC 20 x 20 cm development tank, Vee bottom. (Chromatography chamber)	0.3
25.	4	HPTLC plate x 25, silica, aluminium backed, 20 x 20 cm Merck 5547	0.60
26.	2	TLC plate x 25, silica, aluminium backed, 20 x 20 cm Merck 5553	0.12
27.	2	UV Viewing cabinet, 25x24x22 cm eg. UV Products Model CC10	0.25
28.	2	Lamp, ultra-violet long wave 6W to fit viewing cabinet.	0.28
29.		Lamp, ultra-violet long wave portable battery operated eg UVP No. 95-001-01	0.30

30.	10	Microcaps, disposable micropipettes 5 Ìl (100)	0.03
31.	5	Microcaps, 2 Ìl (100)	0.01
32.	5	Microcaps, 1 Ìl (100)	0.01
33.	2	Box of 400 glass sample vials, 44x19 mm squat Trident vial+ bakerlite screw lids lined with aluminium, 7 ml capacity	0.12
34.	1	Box of glass sample vials, 46x12.5 mm tall + bakerlite screw lids lined with aluminium, 3.5 ml capacity	0.12
35.	2	Boxes of 1000 Pasteur pipettes, unplugged	0.07
36.	20	Bulbs, rubber teats, 1.5 ml	0.007
37.	2	Pipette fillers, pi-pump, 10 ml	0.033
38	1	Pipette filler, pi-pump, 25 ml	0.017
39.	2	Pipettes, Class A graduated, 2 ml	0.07
40.	2	Pipettes, Class A, 5 ml	0.025
41.	2	Pipettes, Class A, 10 ml	0.027
42.	1	Syringe, Hamilton, fixed needle, 100 Ìl. Series 700 sharp point	0.029
43.	1	Syringe, Hamilton, fixed needle 25 Ìl, Series 700	0.025
44.	1	Desiccating cabinet 225x200x168	0.100

C. ELISA equipment

45.	1	Microtitre plate reader, manual (preferably portable with battery option), Filter 410 or 414 nm, to read whole plate.	3.8
46.	1	Pipette, positive (direct) displacement 50 Ìl fixed, eg. Gilson + 1000 disposable tips	0.20
47.	1	Pipette, positive displacement, 100 Ìl fixed, eg. Gilson + 1000 disposable tips	0.20
48.	1	Pipette, air displacement, 5 ml adjustable + 1000 disposable tips.	0.25

49.	1	Pipette, 8-channel, variable volume 50-250 Ìl + 1000 disposable tips.	0.400
50.	1	Resevoir, 8 channel for item 51, pack of 10.	0.04
51	1	Plate shaker (microtitre)	0.75
52.	1	Calculator, scientific, programmable to 50 steps	0.03
53.	2	Timer with alarm and count-down	0.03
54.	1	Flexi-curve for drawing graphs	0.01
55.	1*	Vortex mixer	0.25
56.	4	ELISA kits, total aflatoxin, fully quantitative, eg. Biokits by Cortecs	1.4

D. Safety equipment

57.	5	Gloves, medium, nitrile disposable box of 100 (N-Dex by Best)	0.15
58.	5	Gloves, large, nitrile disposable box of 100 (N-Dex by Best)	0.15
59.	2	Respirator, dust masks, 3M 9920 pack of 10	0.14
60.	10	Spectacles, safety, with side splash protection	0.07

E. Kits and reagents

61.		Assorted rapid immuno-affinity kits, eg. EZ-Screen and Cite Probe	1.5
62.	2	Sodium sulphate, 500 g	0.03
63.	4	Methanol, 25 l drum, AR	0.33
64.	1	Methanol, HPLC grade, 2.5 l	0.011
65.	1	Hexane SLR, 25 l drum AR	0.15
66.	2	Acetone AR 2.5 l	0.018
67.	1	Chloroform, 2.5 l AR	0.033
68.	1	Ethyl acetate, 2.5 l , AR	0.025

69.	1	Xylene, 2.5 l AR	0.023
70.	1	Benzene, 2.5 l AR	0.021
71.	1	Acetonitrile, 1 l AR	0.05
72	1	Trifluoroacetic acid SLR 25 ml	0.021
73.	1	Toluene, 2.5 l AR	0.023
74.	1	Sodium chloride, AR 1 kg	0.015
75.	1	Aflatoxin kit, 1 mg of each standard, B_1, B_2, G_1, G_2. From Sigma AF-1	0.08
Total		US $	29.72

EXCLUDING VAT AND FREIGHT.

* Special price of US$ 4,500 offered by Stephan Machinery UK Ltd., see Annex 2.

NB1. Assumes standards provided by a central laboratory. Otherwise a spectrophotometer, volumetric flasks etc. required. (Novaspec II spectrophotometer from Pharmacia LKB Biotechnology on special offer of 1,100)

NB2. No equipment could be included within the budget to prepare spiked solutions for method evaluation. Need use of sample concentrator etc.

NB3. Assumes that GTS can provide a refrigerator with a freezer compartment, a laboratory oven, a fume-cupboard, a compressor, a water still, air-conditioning and heating, and a reasonable supply of basic glassware such as beakers..

NB4. Items below were removed to meet budget:

12.	1	Voltage stabiliser, 30 A	1.0
60.	1	Ductless fume cupboard with filters to remove organic solvent (TLC) (flow alarms not necessary)	5.0
25.	1	Laboratory oven, 100°C, fan circulated 50 litre eg. Gallenkamp 306.010	0.92

ANNEX 2.

Milling: Cutter Mixer Mill Detailed Specifications.

Stephan Machinery (UK) Limited

Stephan
Food Processing Technology

Unit 7,
Feltham Industrial Estate,
Feltham Way,
Feltham,
Middlesex, TW13 7DP.

Tel: 081-8448202
Tlx: 915044 stephg
Fax: 081-8440988

Banking account: 20637342
Barclays Bank
Heathrow Airport North Branch,
Hounslow, Middlesex.
Code No. 20-38-63

TELEFAX MESSAGE

TO: Natural Resources Institute

ATTENTION OF: Mr Martin Nagler

FROM: Beverley Curr

DATE: 11.11.93

Page: 1

No. of Pages: 2

Fax No: 0634 880066

Further to our telephone conversation, we have pleasure in enclosing details of our machine type UM 25 as requested.

Should you require any further information please do not hesitate to contact us.

Best regards

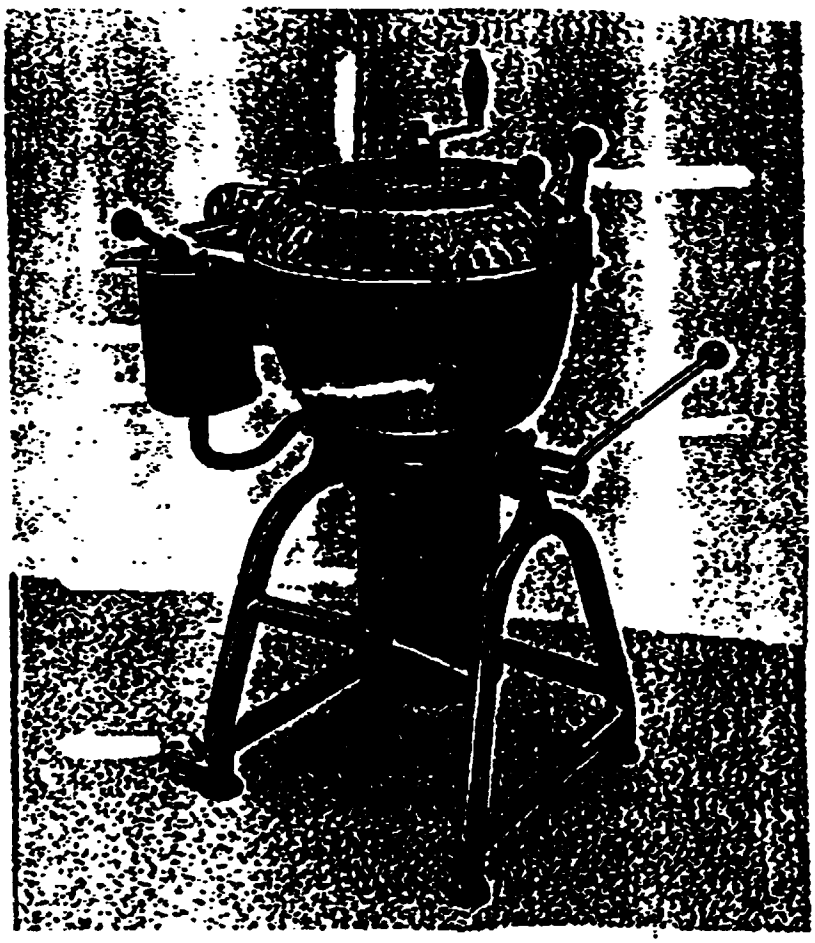
Beverley Curr

Beverley Curr

Enc/.

Stephan Machinery (UK) Limited

Stephan
Food Processing Technology



SPECIAL OFFER — OLD STYLE UM 25 — K.710.332-01

We have available three UM 25 machines at a price of £3,000.00 nett these machines have been modified to meet the Health and Safety requirements of today.

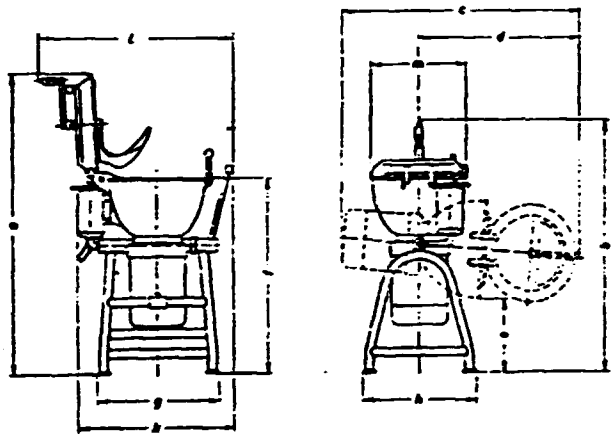
DETAILS OF UM 25

- Bowl contents approximately 25 litres
- Speed 1500/3000 rpm
- Working tools - 2 wide knives
- On/Off push buttons plus emergency stop button
- Electronic brake, wall mounted
- Construction:

  Cover aluminium with inspection opening, chassis, motor and switch housing covered in grey plastic, bowl stainless steel.

Contents

* The Parts List and the Specification cover all available sizes, designs, and models of the Universal UM 25/40. For specific data referring to your particular machine, please refer to the nameplate shown on the inside front cover (this is identical with the data on the actual nameplate fitted to the motor cover).



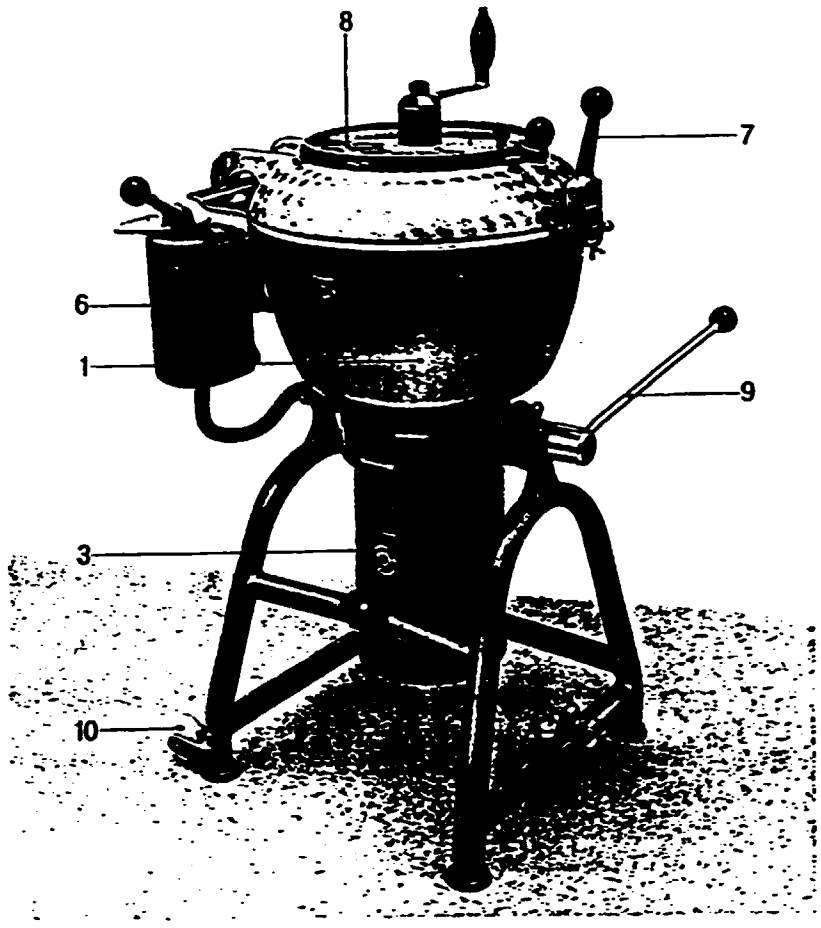
Type	a	b	c	d	e	f	g	h	k	l	mO
UM 25	1400	1160	1190	810	380	900	550	580	800	920	485
UM 40	1600	1200	1360	910	280	915	550	520	850	1000	550

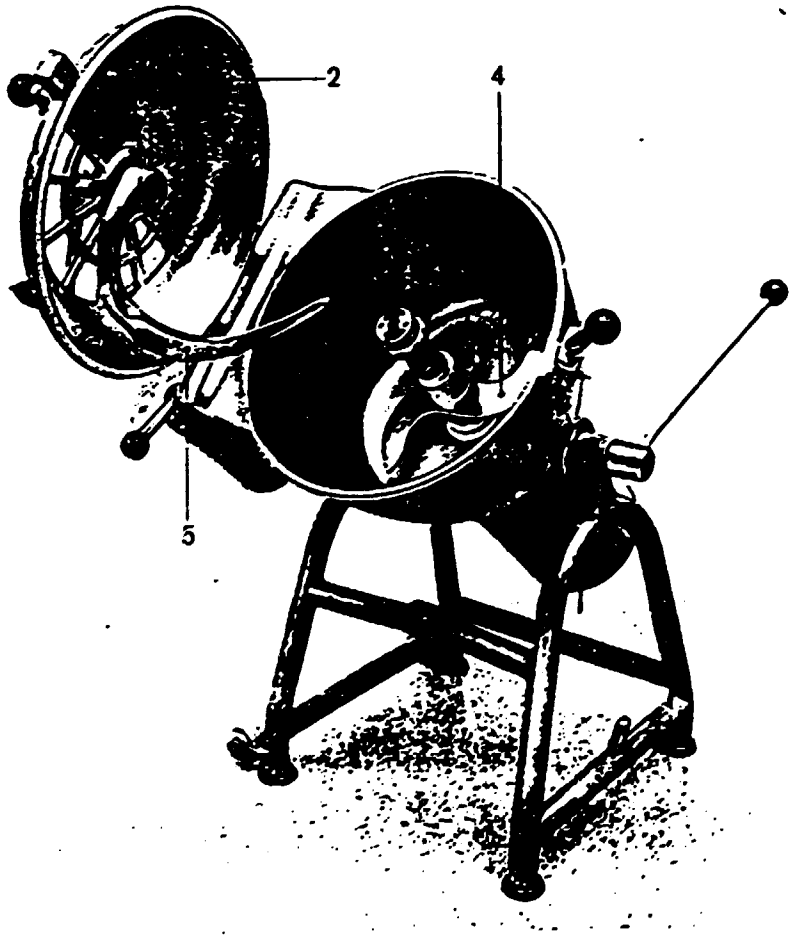
1. Specification

Stephan Universal Machine		UM 25	UM 40
Bowl capacity	litres	25	40
Load at 1450 rpm	kW	3.3	4.0
Load at 2900 rpm	kW	4.0	5.5
Current consumption for a rated capacity of 2.2/3.0 kW 220 Volt	A	17.5	20
Current consumption for a rated capacity of 2.2/3.0 kW 380 Volt	A	10	12
Connected fuses (delayed action) 220 Volt	A	25	35
Connected fuses (delayed action) 380 Volt	A	20	25
Height	mm	1200	1230
Width	mm	770	830
Depth	mm	560	620
Weight	kg	132	138

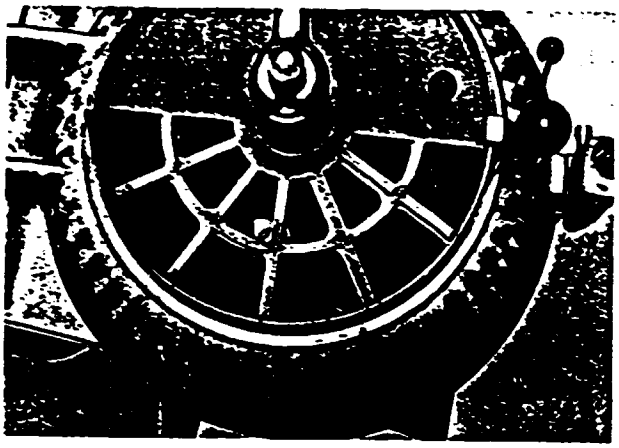
Description and Operating Instructions

1 Bowl
2 Lid
3 Motor
4 Processing inserts
5 Mixing baffle
6 Motor switch
7 Locking lever
8 Inspection grille
9 Tilt-lock lever
10 Castors





2. Installation and commissioning



Due to its weight and smooth operation, the Universal Machine needs not to be fixed to the floor. If it is proposed to operate the machine in a mobile working environment (e.g. on a ship or vehicle etc.), a special tubular stand with screw-down plates can be supplied. Before connecting plug to mains, check that the supply voltage corresponds with that shown on the motor nameplate.

The machine is designed for clockwise operation only. See arrow on motor housing.

If it rotates in an anti-clockwise direction, interchange two of the phase wires at the mains plug. The direction of rotation can be checked by briefly switching on the machine without inserts and opening the lid shortly before the machine comes to rest. The motor shaft should be rotating in a clockwise direction.

3. Functions and descriptions of machine components

3.1 Bowl

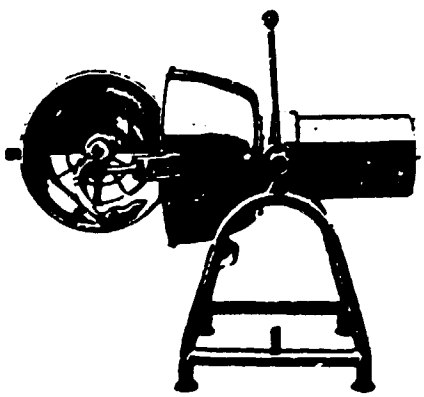
The bowl constitutes the processing area of the machine into which any product, whether solid, liquid or powder, can be loaded.

After the process is completed the bowl can easily be emptied by tilting it to the horizontal.

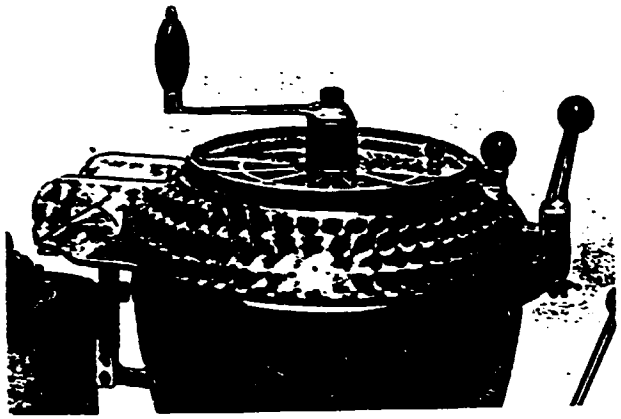
The shape of the bowl and its particularly smooth sides permit the interior to be scraped clean without trouble.

Operating position without accessories

Tilted position



Discharge position



3.2 Lid

The lid serves to:

a) enclose the processing area,

b) protect against accidental contact with the rotating processing inserts,

c) carry the mixing baffle,

d) permit inspection of the process while the machine is running,

e) permit the addition of liquid or powder ingredients during processing.

When the lid is open, the motor cannot be switched on. While the motor is running, the lid cannot be opened.

3.3 Motor

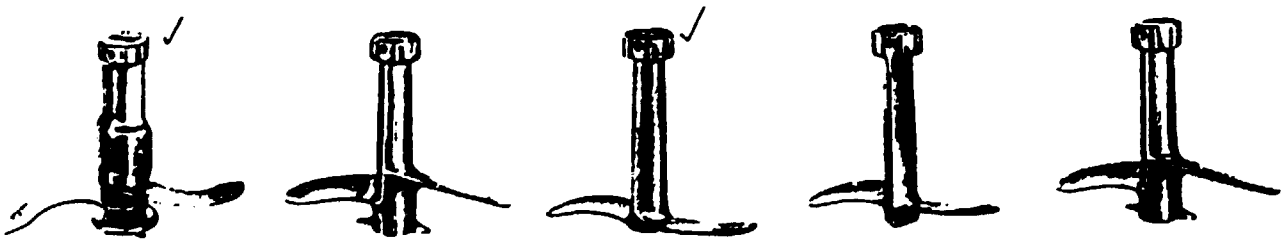
The flanged motor is situated directly below the bowl, and its shaft carries the processing inserts which can be easily removed.

Avoid using force which could result in distortion of the motor shaft.

The motor shaft can also be damaged if tools or other items are accidentally left inside the machine when the motor is switched on.

3.4 Processing inserts

These inserts are the result of many years experience. Every detail has been tested in numerous trials resulting in constant improvements.
Provided, machine and processing inserts are treated with care, very good operating results can be guaranteed for many years.



Cutter | Mixer | Stirrer | Stirrer/Kneader | Grater

ANNEX 3.

Biokits Total Aflatoxin Assay: Outline procedure and results.

CORTECS DIAGNOSTICS
TOTAL AFLATOXIN ASSAY

SAMPLE PREPARATION AND EXTRACTION PROCEDURE

Summary Flow Chart :

Reduce sample to achieve maximum practical size reduction using a suitable mill/blender.

:

Weigh out sample into blender bowl or extraction flask.

:

Add 5ml/g Acetonitrile:H₂O (50:50) to the milled sample in blender/flask.

:

Blend for 2 minutes or stopper and seal extraction flask with tape and place on wrist-action shaker for 30 minutes.

:

Filter resultant slurry and collect filtrate.

:

Prepare 1/25 dilution of extract by adding 0.1 ml of filtrate to 2.4ml of Working Diluent Solution and mix well.

:

Diluted extraction filtrate ready for Total Aflatoxin enzyme immunoassay.

CORTECS DIAGNOSTICS
TOTAL AFLATOXIN ASSAY

ENZYME IMMUNOASSAY PROCEDURE

Summary Flow Chart:

PROCEDURE	VOLUME	DESCRIPTION
addition	50 ul	pipette pre-diluted AFLATOXIN STANDARDS Control Solutions or diluted extraction filtrates
addition	50 ul	dispense RAT ANTI-AFLATOXIN into appropriate assay wells
mix/incubation	:	incubate two (three) hours at room temperature with constant mixing (static) or overnight at 2-8 °C
wash	-	wash 5 times with Working Wash Solution using NUNC 8 probe microwell washer (each wash = 400 ul/well)
addition	100 ul	dispense ANTI-RAT PEROXIDASE CONJUGATE into appropriate assay wells
mix/incubation	:	incubate 30 (60) minutes at room temperature with constant mixing (static)
wash	-	wash 5 times with Working Wash Solution using NUNC 8 probe microwell washer (each wash = 400 ul/well)
addition	100 ul	dispense Working ABTS Solution into each assay well
mix/incubation	:	incubate at room temperature with constant mixing (static)
addition	50 ul	dispense STOP SOLUTION into each assay well
mix	:	mix for 10 seconds on shaker or by hand
absorbance measurement	READ	measure absorbance value of each assay well using plate reader or spectrophotometer

BioKits — **Total Aflatoxin Assay**

QUANTITATION OF TOTAL AFLATOXIN (B_1 G_1 B_2 G_2)

Operator: VICTOR F. REDLOCK Date: 9/11/93

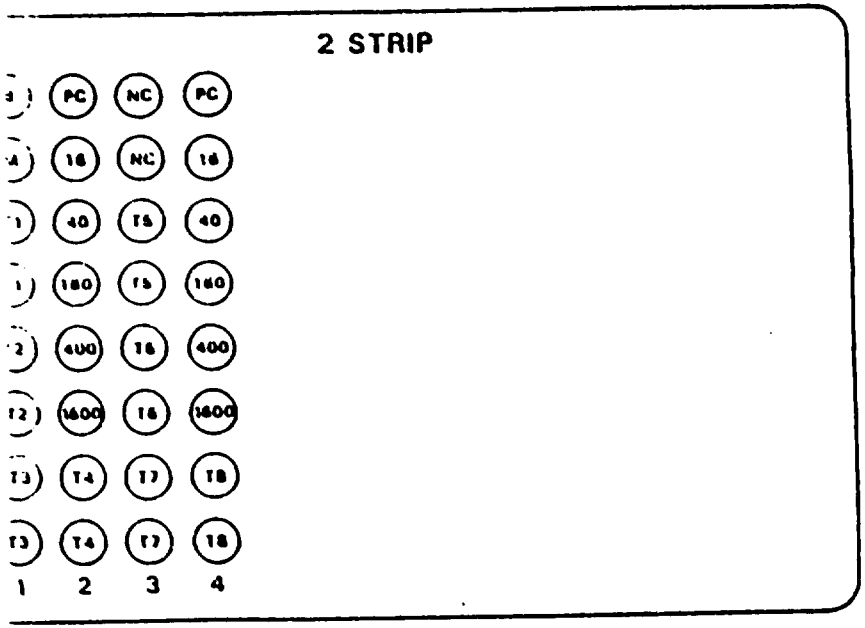
SOLUTION TESTED	ABSORBANCE (414nm)			
	1	2	3	MEAN
16 pg/ml STANDARD	1·68	1·75		1·715
40 pg/ml STANDARD	1·46	1·61		1·535
160 pg/ml STANDARD	0·86	090		0880
400 pg/ml STANDARD	0·47	0·55		0·510
1600 pg/ml STANDARD	0·33	0·25		0·290

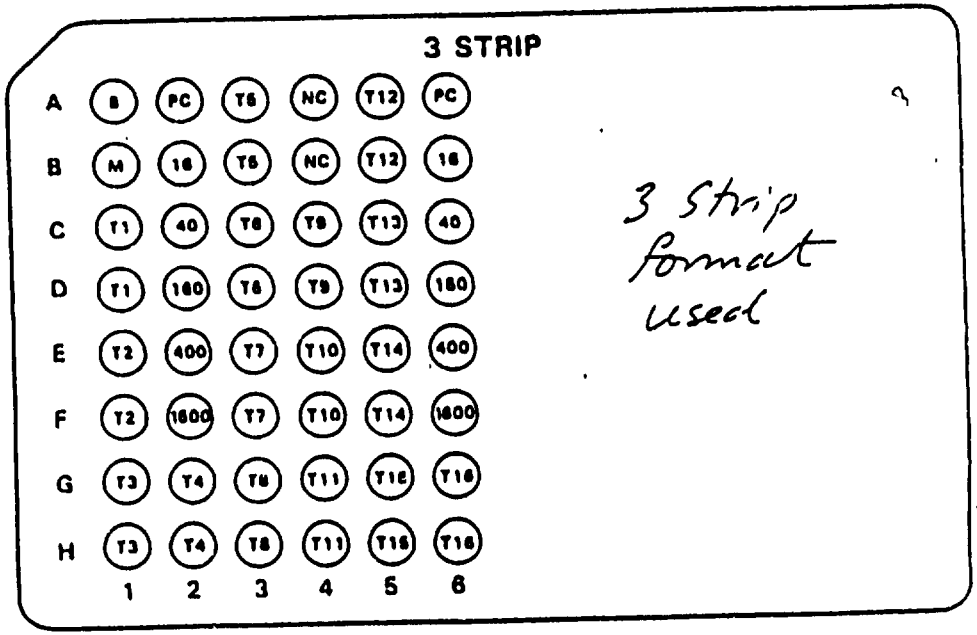
Blank = 0·15

SOLUTION TESTED		1	2	3	MEAN	AFLATOXIN CONTENT OF SAMPLE FROM CALIBRATION CURVE (ppb)
M	MAXIMUM BINDING	1·63				
NC	NEGATIVE CONTROL	197	1·92		1·945	
PC	POSITIVE CONTROL	1·18	1·35		1·265	9·65

SAMPLE IDENTIFICATION			1	2	MEAN	AFLATOXIN CONTENT OF SAMPLE FROM CALIBRATION CURVE (ppb)
1	T1	PK 4	1·42	1·57	1·495	5·6
2	T2	" 10	1·31	1·32	1·315	8·7
3	T3	" 20	0·96	0·91	0·935	18·0
4	T4	" 50	0·64	0·62	0·63	34·0
5	T5	" 200	0·33	0·33	0·33	130·0
6	T6	PN 4	1·79	1·79	1·79	-ve
7	T7	" 10	1·49	1·49	1·49	5·7
8	T8	" 20	1·18	1·12	1·15	12·0
9	T9	" 50	0·72	0·77	0·745	26·0
10	T10	" 200	0·42	0·43	0·425	72·0
11	T11	PK 2	2·0	2·0	2·0	-ve
12	T12	PN 2	1·94	1·89	1·915	-ve
13	T13	PK 0	1·99	1·98	1·985	-ve
14	T14	PN 0	1·99	2·0	1·995	-ve
15	T15	Cortecs glucks	1·87	1·96	1·915	-ve
16	T16	"	1·88	1·92	1·90	-ve

BioKits — Total Aflatoxin Assay





3 Strip format used

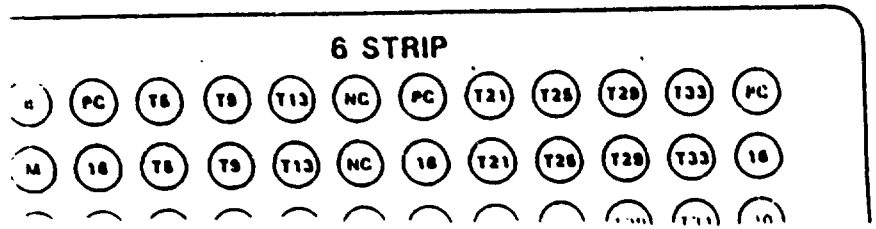
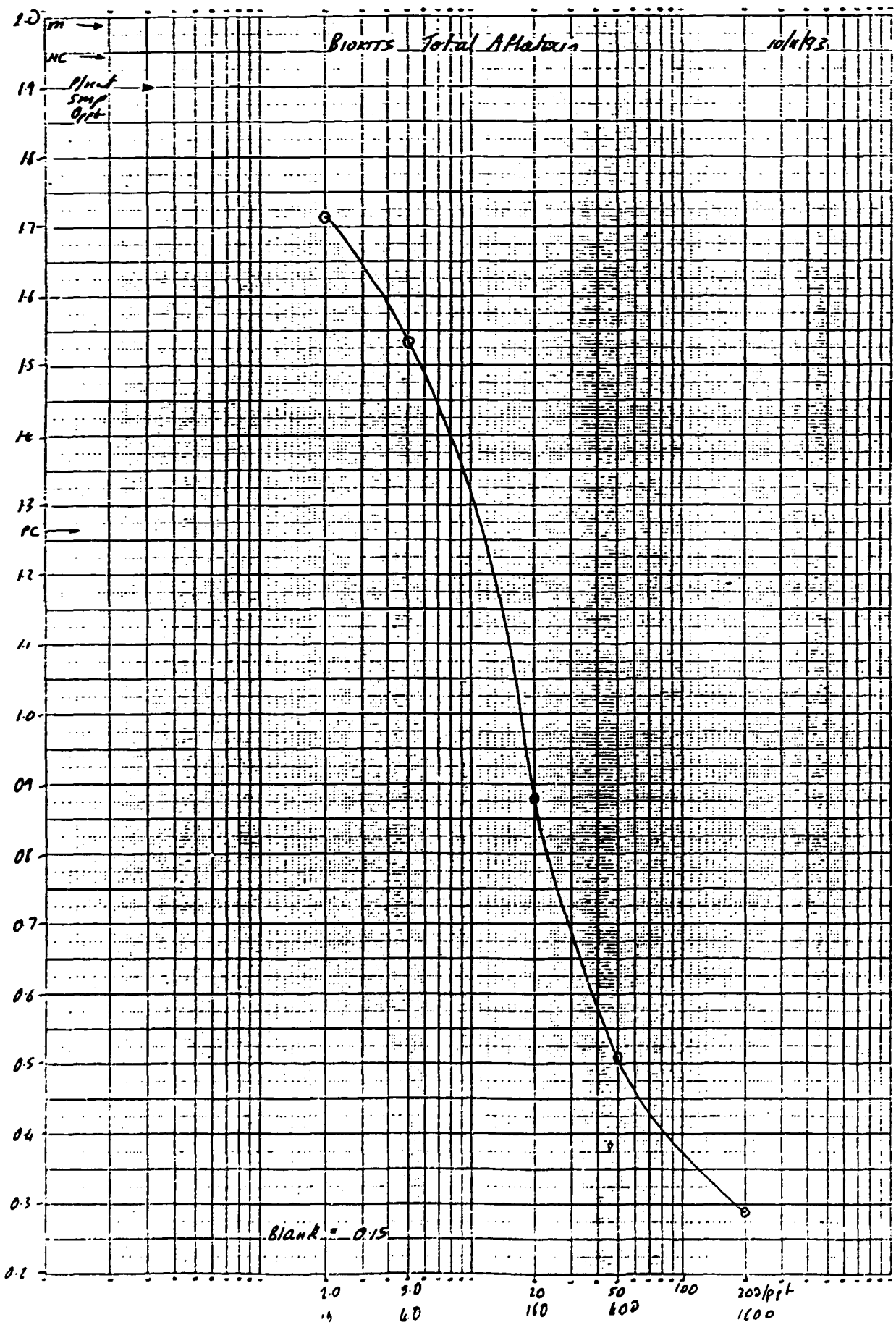
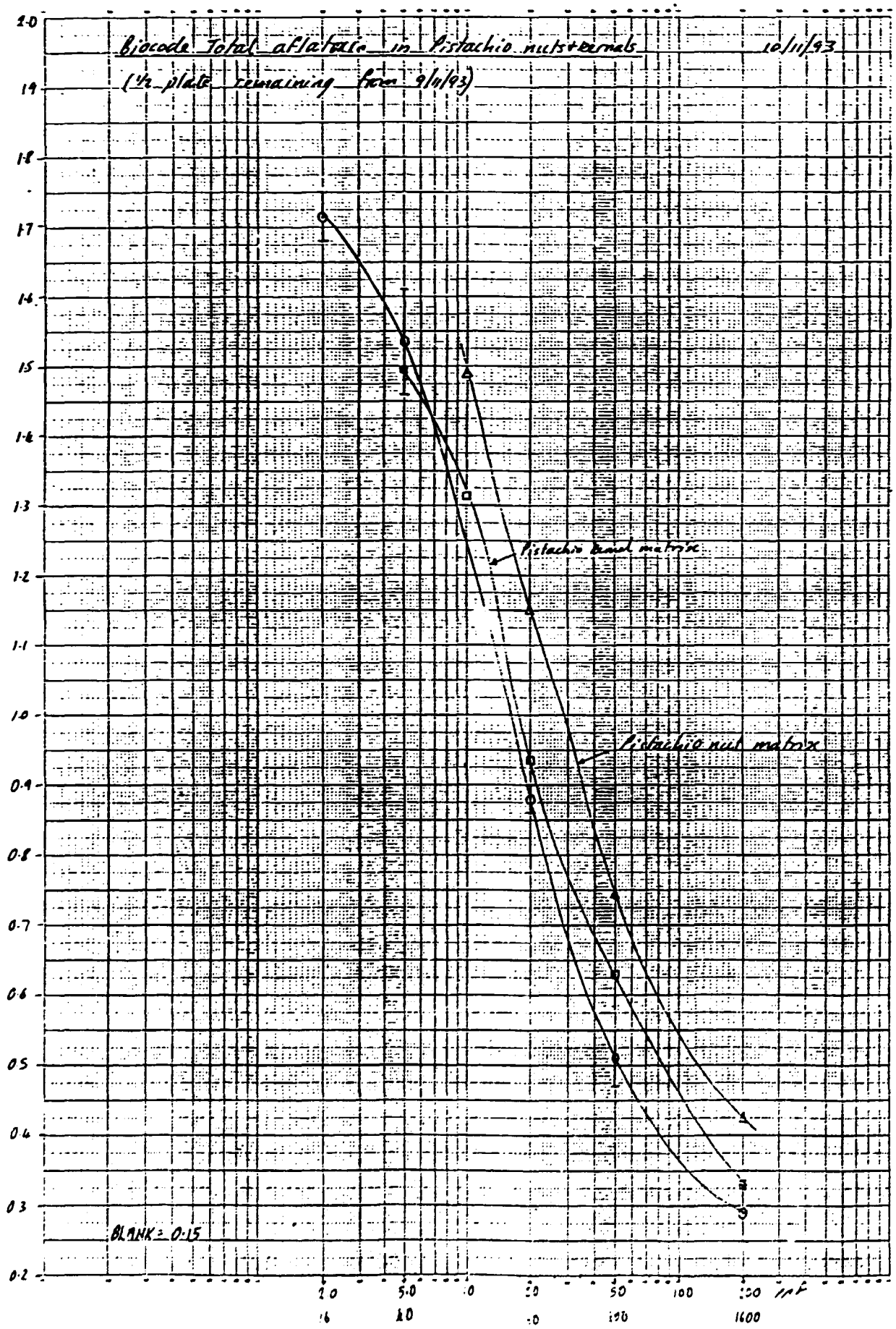


Figure 1
Example Assay Layouts:


Biokits Total Aflatoxin
10/8/93
m
NC
Plant Smpl Opt
PC
2.0
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
Blank = 0.15
2.0
4.0
5.0
10
20
160
50
400
100
200 ppt
1600


Biocode Total aflatoxin in Pistachio nuts+kernals
10/11/93
(½ plate remaining from 9/11/93)
Pistachio kernel matrix
Pistachio nut matrix
BLANK = 0.15
2.0
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
2.0
5.0
10
20
50
100
200
nM
16
40
80
160

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