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ASSISTANCE TO THE PISTACHIO PROCESSING SECTOR

SI/TUR/92/801/11-01

TURKEY

**Technical report: Establishment of International Aflatoxin  
and Food Analysis Laboratory\***

Prepared for the Government of Turkey  
by the United Nations Industrial Development Organization,  
acting as executing agency for the United Nations Development Programme

*Based on the work of M. J. Nagler,  
mycotoxin consultant*

Backstopping Officer: S. Miranda da Cruz  
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United Nations Industrial Development Organization  
Vienna

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\* This document has not been edited.

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## EXPLANATORY NOTES

Exchange Rate.

The UN operational rate of exchange during this mission was:

US\$ 1 = TL 41.900 (part 1)

US\$ 1 = TL (part 2)

Glossary of Abbreviations and Terms.

Aflatoxin	A group of toxins mainly produced by the moulds <i>Aspergillus flavus</i> & <i>A. parasiticus</i> the most abundant & toxic of which is aflatoxin B <sub>1</sub> . This is one of the most potent chemical carcinogens known as well as being an acute poison and immunosuppressive. Total aflatoxin is the sum of the quantities of aflatoxins B <sub>1</sub> , B <sub>2</sub> , G <sub>1</sub> , and G <sub>2</sub> .
AOAC	Association of Official Analytical Chemists
BTOR	Back To Office Report
ELISA	Enzyme-linked immuno-sorbent assay. Advanced technology assay techniques available as packaged kits for aflatoxin testing.
EN45001 (1989)	European standard 'General criteria for the operation of testing laboratories'. Identical to British Standard BS7501 (1989).
FAPAS	Food Analysis Performance Assessment Scheme, organised by the Ministry of Agriculture Fisheries and Food, UK.
FQL	Food Quality Laboratory under the Ministry of Agriculture [il Kontrol Laboratuuar Mudurlugu].
GLC	Gas Liquid Chromatography.

GTS	Government intervention agency supporting farm-gate prices, under the Department of Industry. [Güneydogu Tarim Satis/Güneydogubirlik].
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.
Mycotoxin	General term to describe toxic secondary mould metabolites which are toxic to humans and animals. Aflatoxin B <sub>1</sub> is one of over 300 known mycotoxins.
NRI	Natural Resources Institute, Chatham, Kent ME4 4TB, UK. Scientific agency of the Overseas Development Administration.
ppb	Parts per billion, or ug/kg. A unit of measurement of one part in 10 <sup>9</sup> .
ppm	Parts per million, or mg/kg. One part in 10 <sup>6</sup> .
TFA	Tri-fluoroacetic acid used in aflatoxin confirmatory test.
TUBITAK	Marmara Scientific and Industrial Research Institute, Kocaeli, Turkey. Has a well respected mycotoxin laboratory.
UNIDO	United Nations Industrial Development Organisation

## ABSTRACT

ASSISTANCE TO THE PISTACHIO PROCESSING SECTOR  
SI/TUR/92/801/11-01

Technical report: Second mission of the mycotoxin  
consultant, 24 April-6 May 1995  
and 12-24 June 1995

This report describes the second mission of the mycotoxin consultant which aimed to: commission a pistachio nut aflatoxin quality control laboratory at the Guneydogu Union of Agricultural Sales and Cooperatives (GTS); establish standard operating protocols; and provide training in aflatoxin analysis.

The International Aflatoxin and Food Analysis Laboratory, Gaziantep, governed by an independent Board, was established by the author during this visit. Equipment provided by UNIDO, plus equipment funded locally, was fully commissioned and those items not to specification were noted and replacements requested from the supplier.

Staff were trained and written standard operating procedures were established enabling the laboratory to perform aflatoxin screening by TLC to detect samples of pistachio and red pepper exceeding 1  $\mu\text{g}/\text{kg}$  aflatoxin B<sub>1</sub> and also provide semi-quantitative and quantitative results. Staff were also trained to use various aflatoxin analysis kits.

A 3-day aflatoxin training course was organised and held for 20 analysts working in South East Anatolia.

Recommendations were made to enable the laboratory to satisfy European EN45001 standards and to develop into an internationally recognised, independent aflatoxin testing centre.

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## INTRODUCTION

The Turkish Government requested UNIDO to assist the Pistachio Processing Sector in May 1990. In response, the UNIDO Country Director visited the major pistachio production regions of Gaziantep and Nizip in south Eastern Turkey and presented a BTOR dated 17 July 1990. Following reconfirmation of the Government request in July 1992 this project SI/TUR/92/801 was approved with a UNIDO input of US\$ 98,000.

The UNIDO Country Director revisited Gaziantep and Nizip over 7-9 April 1993 (File Note dated 20 April 1993) to lay the groundwork for project activities, including preparing for the first part of a split mission by the author, acting as a UNIDO mycotoxin consultant.

The UNIDO mycotoxin consultant visited Gaziantep for a two week period commencing 12 July 1993. The associated UNIDO report contained an appraisal of the pistachio industry and also recommended aflatoxin quality assurance procedures and assessed possible locations for the proposed UNIDO aflatoxin laboratory.

Subsequently, UNIDO awarded the Natural Resources Institute a two-week contract to evaluate the most appropriate set of equipment and methods to be used by a laboratory responsible for the determination of aflatoxin in pistachio nuts. The report on this work, including a detailed equipment list, was submitted to UNIDO on 12 November 1993.

There followed a series of delays in: the procurement of the equipment; in clearing it through Customs; and in final delivery to GTS in Gaziantep. Finally, the mycotoxin consultant was able to commence his second mission, of one month duration, on 24 April 1995. This second mission aimed to commission a pistachio nut aflatoxin quality control laboratory at the Guneydogu Union of Agricultural Sales and

Cooperatives (GTS), establish standard operating protocols, and provide training in aflatoxin analysis. The full job description for the mycotoxin consultant, is given in Annex 1.

This report was written by Mr Martin Nagler, the mycotoxin consultant, and describes the second mission referred to above. On arrival in Gaziantep it was learnt that a 10-day religious holiday clashed with the final two weeks of the mission. Consequently, it was decided to split the mission and UNIDO, Vienna, agreed to this proposal. The original dates were 24 April to 19 May 1995 and the revised dates were 24 April to 6 May and 12 June to 25 June 1995 inclusive.

The objectives were fully attained. However, the mission proved more difficult than anticipated due to the fact that the laboratory manager, and the only fluent English speaker at GTS, was recovering from a serious illness which severely effected his performance. In fact, he should really have been recuperating at home, but he insisted on trying his best to perform his managerial and counterpart duties and his endeavours were greatly appreciated.

## I UNIDO AFLATOXIN LABORATORY, GAZIANTEP

### A. Organisation and staffing

A nine-man Board, presided over by Mr Hasan Ozturkmen (President of GTS), has been set up to govern the new UNIDO aflatoxin laboratory at Gaziantep. The following organisations are represented on the Board: GTS (President + 1 member), Gaziantep Chamber of Commerce (2 members), Exporters Union, Grain Market Exchange, Pistachio Research Institute, University of Gaziantep, and the National Project Director (Prof. Fatih Yildiz).

The laboratory, which has been named the International Aflatoxin and Food Analysis Laboratory, is located within the GTS processing factory in Gaziantep. To gain international recognition, and to satisfy European EN45001 standards, the laboratory MUST be (and must be seen to be) independent of commercial organisations with interests in the analytical results. Clearly the laboratory needs to be distanced from its host organisation, GTS, and the setting up of a governing board goes a good way towards this. However, independence might be seen to be compromised by having the president of GTS also holding the presidency of the Laboratory Board. Laboratory staff also need to be independent of GTS and it is recommended that staff be hired directly by the Board.

A laboratory manager, Mr Fatih Turnalar, was appointed by GTS about eighteen months ago. He has been responsible for designing the new laboratory, which has been converted from existing offices adjacent to the factory's quality control laboratory. Most unfortunately, Mr Turnalar suffered a stroke six weeks prior to this visit which left him partially paralysed down his right side. He is recovering slowly and was able to assist with translations. He should, in due course, be capable of carrying out his managerial duties.

Staffing levels and organisation of laboratory staff needs to be finalised. To do this assumptions need to be made concerning the number of samples which will be submitted to the laboratory and the type (screening, semi-quantitative, or quantitative) of analysis that will be required. It would be realistic to plan initially for 50 samples a week with half of these requiring quantitative analysis. In this case the laboratory would require:

Laboratory manager: responsible for the validity of the result; ensures that all SOPs are being rigidly adhered to; records incoming samples and analytical results; despatches reports; manages analysts; maintains laboratory (eg orders consumables and arranges for repairs); liaises with reference laboratories; implements quality assurance; and interfaces with clients.

Senior analyst: responsible for all practical laboratory work including preparation determination and maintenance of standards, sample preparation, sample extraction, clean-up, work-up and chromatography. Also responsible for day to day planning of work and acts as line manager for junior staff.

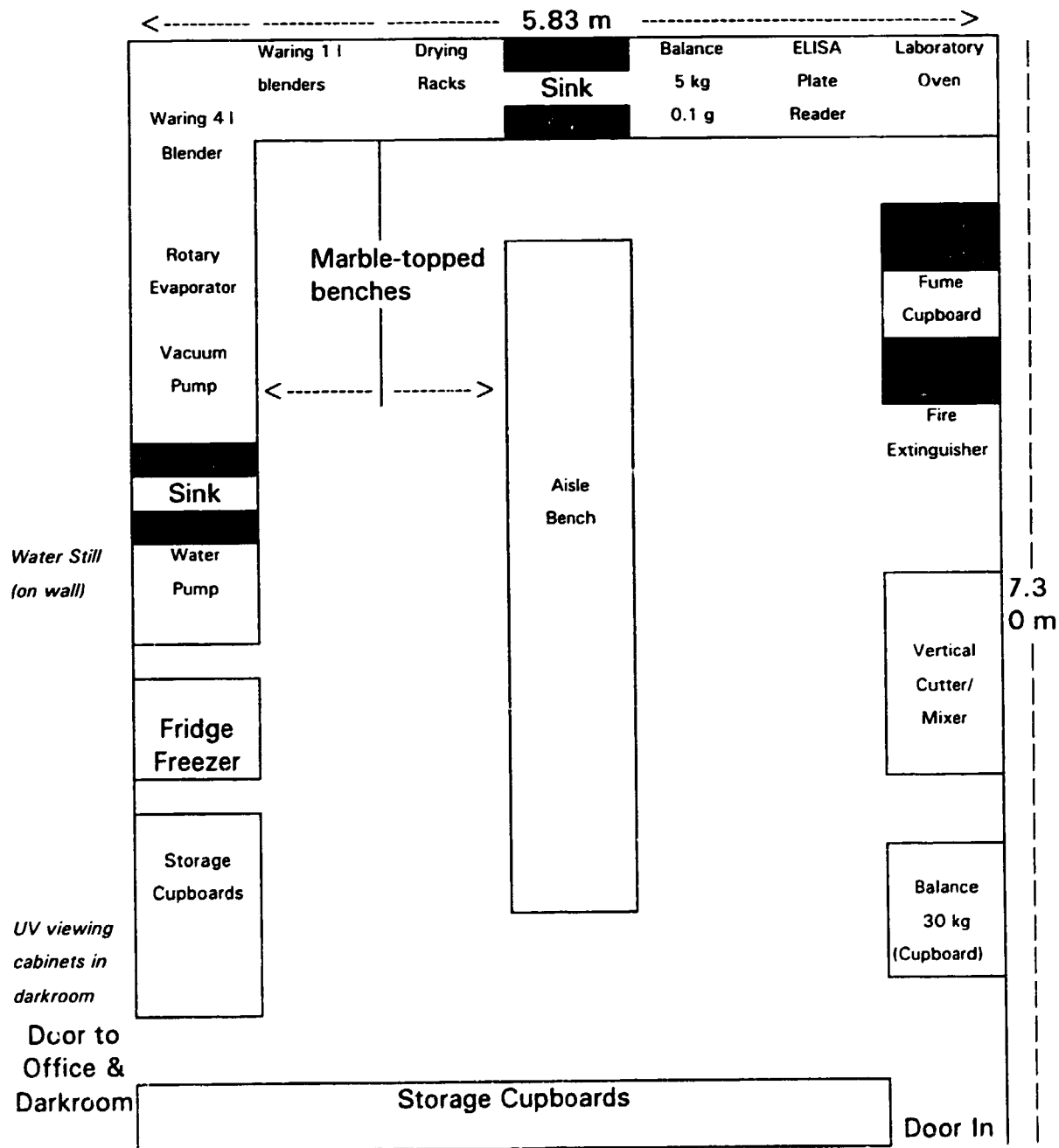
Junior analyst: assists the senior analyst in all practical duties. Should act as stock manager, alerting senior staff when stocks of consumables need re-ordering.

Laboratory worker: performs sample preparation duties, washes-up laboratory glassware, tidies laboratory daily.

**B. Laboratory design & modifications**

A large room had been converted into a well-planned laboratory with marble benches, two sinks, and a fume-hood, as shown in Figure 1 below.

**Figure 1. Design of the UNIDO Aflatoxin Laboratory, Gaziantep**



In addition to the main laboratory there was a large office and a small darkroom. The office was equipped with two desks, a computer, a telephone and a fax machine. The darkroom housed the UV-viewing cabinets.

The following recommendations were made, all of which were implemented during the visit: painting of external windows to exclude direct daylight; installation of tungsten lighting to replace fluorescent tubes; addition of a sliding front panel to the fume cupboard to improve efficiency, installation of a water pump to improve pressure.

The following items of laboratory equipment were recommended to be purchased locally: oven; water still, fridge/freezer. All items were in place for the second part of the split mission.

#### C. Commissioning of equipment

The vertical cutter/mixer mill had been installed, but no other items of UNIDO equipment had been un-packed prior to the consultant's arrival.

Each item of equipment was checked against the equipment list, and it was then commissioned and allocated a position for operation and/or storage within the laboratory suite.

A number of items of equipment supplied by UNIDO did not meet the required specification:

- i) a rotary vacuum pump and helium cold traps had been supplied instead of a simple, but solvent tolerant, diaphragm pump;
- ii) air displacement pipettes had been supplied instead of direct displacement pipettes;

- iii) poor quality separating funnels had been supplied with ill fitting stoppers and taps;
- iv) the ELISA plate reader did not have a power supply and the plate holder was ill-fitting;
- v) the Easi-Assay kit had been shipped when already out of date;
- vi) the four expensive ELISA total aflatoxin kits had been shipped with no special instruction for refrigerated, or these instructions had been ignored. Four months storage at ambient temperatures in Turkish Customs had completely destroyed the kits.

UNIDO was notified and all these matters were raised with the suppliers, LABQUIP. LABQUIP has promised to replace items; i), ii), iii), and v) and the manufacturers of the ELISA plate reader have provided the missing power supply. Nobody has accepted responsibility for the ELISA kits and these may have to be written off.

#### D. Safety in the Aflatoxin Laboratory [SOP1]

A safety protocol was drawn-up with the agreement of the laboratory staff. This protocol, designated standard operating procedure number one (SOP1), was translated into Turkish and displayed prominently on a laboratory wall. The English version is given in Annex 3A.

The protocol was rigorously adhered to during the visit.

### E. Aflatoxin standards [SOP2]

Aflatoxin standards (Sigma) were provided by UNIDO, but had not been stored at 0°C as recommended by the manufacturers, during transit and storage prior to this consultants visit. The integrity of the standards was, therefore, in some doubt, but ambient temperatures had not been high over the winter months and fortunately the standards were found to have survived.

The aflatoxin standards were prepared and the concentrations accurately determined at the University of Gaziantep, thanks to the kindness of Prof. Mehmet Öner who offered the facilities. Training in these procedures was given to Mrs Aysel Kasakolu from the UNIDO laboratory and Dr Fahrettin Gögüs, lecturer at the University of Gaziantep.

A standard operating procedure (SOP2) was drafted and followed, see Annex 3B. Fortunately, the University had a Novaspec II UV single-beam spectrophotometer which was ideal for the job when working in the 'peak check' mode. Results are summarised in Table 1 below:

Table 1. Determination of the concentration of aflatoxin standards

AFLATOXIN	B1	B2	G1	G2
Batch Number	LOT 123H4039	LOT 92H4021	LOT 13H4027	LOT 83H4090
Solvent	Bz:ACN	Bz:ACN	Bz:ACN	Bz:ACN
Wavelength $\lambda_{max}$ (350)	349	350	355	357
Absorbance at $\lambda_{max}$	0.6435	0.507	0.507	0.5515
Molecular Weight	312	314	328	330
Correction Factor	1*	1	1	1
Molar Absorptivity	19,800	20,900	17,100	18,200
Concentration (Calculated)	10.14	7.617	9.725	10.00



\* Correction factor assumed to be one.

Bz:ACN = benzene:acetonitrile 98:2

Concentration (c) is given by the equation:

$$c = \frac{ABS \times MWt \times CF \times 1000}{E \times L},$$

where ABS is the maximum absorbance recorded for the particular toxin, MWt its molecular weight, E its molar absorbance and L the cell path length, (in this case L = 1). CF is the correction factor, if any, for the spectrophotometer.

The molecular weights and molar absorptivities are literature values and can be substituted into the equation directly. The correction factor and absorbance can be determined by following SOP2 allowing the concentration of aflatoxin to be calculated.

The standards had the correct  $\lambda_{max}$  values and the concentrations were close to the nominal 10  $\mu\text{g}/\text{ml}$  which would be expected if 1 mg exactly of standard had been provided. The concentration of aflatoxin B<sub>2</sub> was somewhat lower than the nominal value, but this is often the case for this toxin because it dissolves only slowly in Bz:ACN. A 'mixed' working standard containing all four aflatoxins was then prepared by diluting the above 'UV-Standards' by a factor of twenty. These standards were kept in a freezer at minus 18°C and protected from daylight.

Each vial of working standard should be used to spot a maximum of five TLC plates, as stipulated in the 'Confirmation and Quality Assurance' protocol, SOP4, see Annex 3D. UV-standards should be re-determined every six months, prior to the preparation of a new batch of working standards.

## F. Sample preparation protocols [SOP3]

### Pistachio kernels

Sample preparation protocols were devised for both pistachio kernels and pistachio nuts. Both methods utilised the vertical cutter/mixer which was bought specifically for this purpose. This equipment enabled a large 10 kg laboratory sample to be milled and thoroughly mixed prior to removing a representative 80 g analytical sample.

Two approaches were tried when preparing samples of kernels. In the first a coarse, fairly free flowing, material was produced and 1 kg of this was homogenised with water (1:1.5) to produce a slurry. In the second a homogeneous paste was prepared and an 80 g aliquot was taken directly as the analytical sample.

Analytical samples prepared by both of these procedures were analysed by the 'Steiner' TLC method, see below, and it was found that the paste resulted in a much 'cleaner' extract than the slurry when subjected to TLC. It was therefore decided to use the 'paste' procedure, even though the slurry might well have given the more homogeneous analytical sample.

The SOP which describes the production of pistachio paste is given in Annex 3, SOP3, Section A, Milling.

### Pistachio Nuts in shell

A procedure was devised whereby the whole nuts were first crushed using the 'stirring/kneading' attachment fitted in the vertical cutter/mixer, see Annex 3D, Section B. Milling. The product was then sieved using a 2 mm screen to separate the shell from the kernel. The kernel fraction, which accounted for about 45% by weight of the whole nut, passed

through the sieve and was then formed into a paste as described above.

**G. The TLC method protocol [SOP3]  
(screening and quantitative)**

The TLC method selected was based on the AOAC 'BF' method (Cunniff P. Ed. 1995) which has been officially adopted for the analysis of aflatoxin in groundnuts and groundnut products. Steiner modified the method (Steiner W. E. 1992) to improve extraction and avoid the use of a centrifuge and he showed that it was applicable to the analysis of pistachio nuts. This modified method has been adopted as the SOP for the UNIDO laboratory, see Annex 3D, SOP3.

The 'Steiner' method was selected because it was relatively simple to perform, did not require expensive equipment, had very low running costs, gave clean extracts, and allowed detection down to 1  $\mu\text{g}/\text{kg}$  (ppb) due to the high sample weight in the final extract.

The TLC method was adapted to operate as a screening method working to 1  $\mu\text{g}/\text{kg}$  and as a semi-quantitative method capable of grading samples into the ranges: less than 1; greater than 1 and less than 4; greater than 4 and less than 10; and greater than 10. It could also operate in a quantitative mode using visual comparison with standards.

Aflatoxin contamination of red pepper was causing concern at the time of the visit and the 'Steiner' TLC method was applied to this commodity. Initial tests looked promising and it proved possible to detect low levels of aflatoxin in this matrix. Further tests will be required to confirm the suitability of this TLC method for the analysis of red pepper.

## H. Confirmation and Quality Assurance {SOP 4}.

It is very important to confirm that the fluorescent spots that are quantified really contain aflatoxin. To do this a hemi-acetal derivative is formed on the HPTLC plate prior to development. Aflatoxins B<sub>1</sub> and G<sub>1</sub> form derivatives which run slower than the parent aflatoxins. If these aflatoxins really are present then the parent spots disappear, or become far less intense, and a new slower running spot corresponding to the hemi-acetal derivative appears. Aflatoxins B<sub>2</sub> and G<sub>2</sub> do not form derivatives and therefore can not be confirmed using this method.

At least 5% of all positive samples routinely analysed, and all samples from new sources or of commodities not previously analysed in the laboratory, should be confirmed by SOP4, see Annex 3D.

SOP4 also has a section on quality assurance. Use of reference materials, inter-laboratory checks, and use of spiked samples to check recovery is described. This SOP is designed to detect early if the analytical method is giving erroneous results. If errors are detected then urgent troubleshooting is required to find the cause. The problem could be due to a number of reasons including: failure to adhere to the SOP; faulty standards; and faulty reagents.

### I. Rapid aflatoxin testing kits

Training was given in the use of two rapid aflatoxin kits: Easi-Assay mini-column (Rhone Poulenc) and the EZ Quik-card test (Environmental Diagnostics, distributed by Rhone Poulenc). The EZ Quik-card test was by far the simplest and quickest (10 minutes) of the two tests and did give reliable results for both pistachio and red pepper extracts when working to a 5 µg/kg aflatoxin B<sub>1</sub> limit. The Easi-Assay test

took about 1 hour to complete, and was hard work when a syringe was used. The extra cost and effort would only be justified if the commodity under test could not be analysed by the EZ Quik-card test.

The laboratory's TLC method would be far cheaper to perform than either of the kits, but the kits do not require laboratory facilities and would be ideal for use by traders and exporters to identify suspect batches.

The kits come with a full set of instructions.

#### J. ELISA fully quantitative kit

The laboratory is equipped to carry out ELISA analyses and use of these kits offers an objective method for obtaining quantitative aflatoxin results: TLC is a subjective method. ELISA kits are not robust and need to be imported under refrigeration, which might make supply difficult and hence be a constraint to the routine use of the method in the UNIDO laboratory.

The method involves many steps and it is easy to make a mistake when using this kit. In order to minimise such errors, a bench-overlay designed at NRI was provided. Laboratory staff were given training and should be able to perform the analysis competently.

Although it takes about four and a half hours to complete, it is possible to quantify up to 37 extracts in this time, making it very fast per sample when a high throughput of samples is required. It is more usual to split the kit into three and analyse 8 samples at a time.

### K. Future laboratory requirements

Currently the laboratory is dependent on the University of Gaziantep for determining accurately the concentration of it's aflatoxin standards. The 'UV-standards' need to be determined about four times a year, prior to being diluted to give a fresh batch of working standards. To avoid this and maximise it's independence the laboratory needs a good quality UV-visible spectrophotometer with quartz 1 cm cells with a tight-fitting stopper. The Novaspec II (Pharmacia) single beam spectrophotometer, as used at the University, is ideal (£2,000).

The laboratory plans to computerise it's records and suitable spreadsheet and database software is required and this needs to be customised. Staff will also require training.

The next major step forward for the laboratory will be the introduction of a more accurate and precise means of quantification. The choice is between HPTLC and HPLC. The former is a relatively simple, robust system which is an extension of the existing TLC system. HPLC has many components, is a less robust system than HPTLC, and requires staff who are highly trained. It is, however, capable of detecting lower levels of toxin than HPTLC and precision is better when aflatoxin is present in the 1 to 10  $\mu\text{g}/\text{kg}$  range. HPLC is also useful for analysing other food contaminants, such as some pesticides, although GLC is still the method of choice for pesticides.

HPLC and HPTLC have similar, high, capital costs, in the order of £25,000. Availability of maintenance, both routine and emergency, must be a major consideration in the choice of technique.

## II AFLATOXIN TRAINING COURSE

A 3-day training course was organised, see programme in Annex 4A. Briefs were supplied to Prof. Öner (Aflatoxin and its significance, Annex 4B) and Prof. Yildiz (Project background/aims of the laboratory) and the following papers were prepared: Methods of Aflatoxin Analysis: Principles and Criteria for Selection, Annex 4C; Sample Collection and Sample Preparation, Annex 4D. Overhead projector slides were prepared, many in Turkish, and some of the talks were translated into Turkish and given as hand-outs.

A total of 20 trainees attended the course and were presented with attendance certificates by Prof. Yildiz. The trainees came from throughout South East Anatolia, not just Gaziantep, and worked in laboratories which provide quality control for a range of commodities including: groundnuts, spices, cottonseed, wheat, and pistachios.

Trainees seemed particularly impressed with the demonstration of the EZ Quik-card test and thought that it would be useful for traders and exporters, who didn't have laboratory facilities, to identify suspect batches.

### III. PISTACHIO PROCESSING

There have been a number of significant changes to the way in which GTS processes pistachios since the last visit by the UNIDO mycotoxin consultant in July 1993.

In 1993 only a very small proportion of pistachios were de-hulled by splitting in a dry state. Now it is estimated that 2,000 MT per year (25%) are processed dry. The process is carried out in cottage industries and the nuts are sorted into whole kernels and split kernels. All kernels marketed by GTS were reported to be produced by the dry process. It is most unlikely that kernels produced by this process would become contaminated with aflatoxin during, or as a result of, processing. Any aflatoxin contamination of kernels would therefore have occurred either pre-harvest or during storage of the nuts in-hull.

The majority of the pistachio nuts, approximately 6,000 MT, is still de-hulled by the wet process, but using new plant. The de-hulling process takes 20 minutes per batch and the resultant nuts in-shell are then washed and dried in a mechanical dryer for a fixed period of time. The nuts are then divided and some are bagged directly and sent for export whilst others are taken for immediate roasting and packaging.

The roasted nuts would be completely dry and therefore safe from aflatoxin contamination. The un-roasted nuts, however, are highly dependent on the efficiency of the dryer. If drying of some of the nuts is incomplete, then they may be susceptible to aflatoxin contamination during subsequent storage and transit. This may constitute a major aflatoxin risk and replicated storage trials should be conducted to monitor both moisture contents and aflatoxin levels during a two-month storage period.



#### IV. CONCLUSIONS

1. The UNIDO aflatoxin laboratory has been commissioned and is ready to analyse samples of pistachio kernels and pistachio nuts by a TLC method, but it does require continuing support.
2. Some important items of UNIDO equipment failed to meet specification, notably the vacuum pump and ELISA kits.
3. The laboratory does not yet meet all the standards set in EN45001 and aspects of organisation, staffing and policy need further attention.
4. Quality assurance protocols have been introduced and an integral part of these is establishing links with reference laboratories in Turkey and overseas in order to participate in check-sample programmes. In order to comply with EN45001 it will also be necessary to participate in an internationally recognised check-sample programme such as FAPAS.
5. Laboratory staff require further training in analytical techniques, laboratory management, computerised record keeping, and determination of standards.
6. Manual spotting of TLC plates was identified as a major constraint in achieving quantitative results and a semi-automatic spotter is required.
7. The arrangement whereby the UNIDO laboratory is dependent on the University of Gaziantep for determination of aflatoxin standards is not ideal and it is preferable to make the UNIDO laboratory fully independent.

8. Aflatoxin surveys are needed to establish the seriousness, or otherwise, of aflatoxin contamination of pistachio kernels produced in Turkey: by the dry process; roasted nuts in-shell; and un-roasted nuts in-shell destined for export.

## V. RECOMMENDATIONS

### *Short-term*

- a) the laboratory should meet European EN45001 standards. It needs: a laboratory policy statement agreed at Board level; staff who are independent of GTS; and clearly defined duties and responsibilities for all laboratory staff;
- b) the laboratory should be given considerable support over the next three years, including further training of staff in Turkey and at overseas centres of excellence, and regular visits by a mycotoxin consultant to check the progress of the laboratory, provide additional training, advise on work programmes, and advise on the development of the laboratory;
- c) a semi-automatic plate spotter, such as a CAMAG Nanomat III, should be purchased;
- d) equipment for the preparation of working standards should be purchased: 500  $\mu$ l Hamilton syringe; 1.5 ml amber vials; volumetric flasks, grade A, 10 ml (x10) and 20 ml (x5);
- e) the laboratory should participate in local and international sample check studies eg the Food Analysis Performance Assessment Scheme (FAPAS);
- f) laboratory records should be maintained on a computer spreadsheet (Excel 5) and a computer database (Access 2) after staff have received adequate training;
- g) an aflatoxin survey of pistachio nuts should be undertaken including regular samples of: kernels produced by the dry process, roasted pistachio nuts in-shell; imported kernels and nuts in-shell; and un-roasted pistachio nuts in-shell destined for export. A

storage study should be carried out on batches of the export nuts in-shell to monitor any development of aflatoxin over a 2-month period.

h) Each vial of working aflatoxin standard should be used to spot a maximum of five TLC plates and UV-standards should be re-determined every six months, prior to the preparation of a new batch of working standards.

*Medium- and long-term*

i) a UV-visible spectrophotometer should be purchased (eg Novaspec II, single beam) and staff trained to prepare and determine aflatoxin standards;

j) Either HPLC or HPTLC equipment should be purchased, and staff trained, to upgrade quantitative capability and expand scope if and when the demand exists (consultant advise should be sought when the upgrade is justified);

## VI. ANNEXES

### Annex 1

#### Job Description

The complete Job Description covering both the previous mission in 1993 and this mission in 1995, is given below:

Duties: In close coordination with the National Project Director (NPD), the expert will be responsible for providing technical advice to the Turkish Authorities in the region of Gaziantep on the improvement of quality of pistachio nuts. He / she will be responsible specifically for the following activities:

1. Technical assessment of the pistachio processing sector in the region (including laboratory facilities at the Gaziantep University.
2. Definition of a detailed list of equipment to be purchased in order to complete the set-up of an adequate pistachio nut analysis laboratory at Gaziantep University.
3. Introduction of the laboratory personnel to the required analytical procedures for pistachio nuts, especially those related to pesticides and mycotoxin / aflatoxin residues analysis.
4. Completion of the installation of the analytical equipment (if required) and jointly with laboratory personnel, conduction of tests in order to consolidate their ability in carrying out pesticides and aflatoxin residues analysis.
5. Implementation of part of a training programme for approximately 30 Turkish technicians and professionals from the Gaziantep region in order to be formally introduced to,

and to be in a condition to carry out analytical tests, for controlling the levels of pesticide and aflatoxin residues in pistachio nuts.

6. The expert is also expected to prepare two reports (one after each phase of the split mission) providing conclusions on the technical assessment of the pistachio processing sector (including laboratory facilities) in Gaziantep and recommendations to be followed by the Government authorities in order to improve the chemical and microbiological quality of the final product.

NB.1. The first mission covered items 1) and 2) of the TOR. Items 3) through 5) were addressed during this second mission and reports were prepared in accordance with TOR 6).

**Annex 2: Programme of Visit**

24 April to 5 May and 11 to 25 June 1995.

Date	Time	Activity
24 04.95	am/pm	Flew to Ankara, met by Prof. Fatih Yildiz, Booked into Best Hotel
25.04.95	09.00	UNIDO office for Admin.
	10.30	Met Prof. Yildiz & visited British Council. Discussed training with Mr A Picken.
	11.30	EU Office to discuss possible funding under Fourth Protocol
	pm	Flew to Gaziantep & met Mr Ahmet Adali (Deputy General Manager, GTS)
	pm	Booked into Tugcan Hotel
26.04.95	am/pm	Met GTS staff & Prof Mehmet Öner/ Birgul Akar of Gaziantep University. Un-packed and located equipment. Faxed UNIDO, Vienna, informing of 10-day holiday and requesting split mission.
27.04.95	am/pm	Commissioned balances and vertical cutter/mixer. Carried out milling trials on kernels & whole nuts.
28.04.95	am/pm	Commissioned rotary evaporator and compared extraction and clean-up procedures for pistachio slurry and paste.
29.04.95	am/pm	Prepared UK aflatoxin standards and demonstrated quantitative TLC using TLC/HPTLC plates using two development systems.
30.04.95	am	Started writing report.
01.05.95		Trained GTS staff: TLC extraction & clean-up
02.05.95	am/pm	Advised on selection of water still & oven for new laboratory. Prepared & determined aflatoxin standards at Gaziantep University.
03.05.95	am/pm	Performed recovery experiments on quantitative TLC method using new standards.
03.05.95	pm	Finalised new itinerary for split mission. Made phone calls to Prof. Yildiz and had discussions with Prof. Öner and Mr Huseyen Senturk.

04.05.95	am	Trained GTS staff and Dr Fahrettin Gögüs in interpretation of quantitative TLC plates and the calculation of results
	pm	Trained GTS staff to perform recovery experiments on the quantitative TLC method.
05.05.95	am	Trained Mr Fatih Turnalar in aspects of TLC and sampling. Demonstrated qualitative TLC method to GTS staff and Prof Öner.
	pm	Supervised GTS staff performing screening TLC procedures
06.05.95	am/pm	Flew back to UK because of long Turkish Holiday
11.06.95	am/pm	Flew Istanbul/Gaziantep
12.06.95	am	Contacted Mr Adali, discussed training, opening ceremony and brochure: agreed
	pm	Met Mr Shenturk and Aysel, discussed progress (no analyses performed!) and was shown new laboratory equipment
13.06.95	am	Concerned about lab. staffing and lack of arrangements for the training course and opening ceremony. Phoned Prof Yildiz
	pm	Red pepper TLC screening extraction, clean-up and work-up. Photographs for the brochure. EZ card test on red pepper demonstrated to GTS + Dr Öner
14.06.95	am	Mr Fatih at work, insisted on translating brochure instead of Dr Fahrettin. Red pepper TLC, good.
	pm	Easi-Assay on red pepper demonstrated: looked promising. GTS decided not to hold lab. opening ceremony on 21.06.95 because 'not ready', training course in doubt. Faxed Prof. Yildiz
15.06.95	am	Discussed Training Course problems with Dr Öner and he convened a meeting of the Laboratory Board at lunch-time. Course confirmed. Prepared for ELISA
	pm	Demonstrated Cortecs total aflatoxin kit, found to be not viable. Plate reader has problem with plate holder, but just usable. (Fatih/Aysel/Fahrettin)



16.06.95	am	Gave remainder of lab. manual method notes, excl. kits, to Fatih/Fahrettin for translation into Turkish. Discussed materials needed for course and GTS purchased these. Fully quantitative TLC of pistachio spiked to 6 and 15 ppb.
	pm	Plate spotting/reading: much more practice required. Easi-Assay on pistachio spiked at 4 ppb: aflatoxin band was clearly visible.
17.06.95	am/pm	Quantitative TLC training, spiked pistachio at 6 ppb. Decanting of sodium sulphate not being performed correctly. Great improvement, good plate.
18.06.95	am/pm	Preparation of talks and acetates for training course presentation.
19.06.95	am	Persuaded Dr Öner to call meeting of the Lab. Board. Agreed to hold course and provide a trainee analyst. University did not agree to do translations
	pm	Training, confirmation of aflatoxin using TFA. More translation of course material into Turkish
20.06.95	am/pm	Translations, typing Turkish text directly into portable computer. Final laboratory preparations for the Course. Prof. Yildiz arrived
21.06.95	am	Course opening ceremonies, 2 x TV channels. Talks on safety in the laboratory, significance of aflatoxin, and analytical methods
	pm	Talk on sampling and sample preparation, demonstration of sample preparation of pistachio kernels, Steiner extraction and clean-up. Calibration of trainees for TLC screening.
22.06.95	am	Demonstration, TLC screening methods. Talk on standard preparation and use.
	pm	Demonstration, quantitative TLC and calculation of results. Talk & demonstration, confirmation of aflatoxin.

23.06.95	am	Talk on ELISA and demonstration of Cortecs total aflatoxin kit.
	pm	Demonstration of EZ-screen quik-card test and Easi-Assay kit. Discussions on selection of methodology Awarding of certificates
24.06.95	am	Equipment inventory with Prof. Yildiz. Recap on SOP2, procedures for determining the concentration of standards.
	pm	Setting-up laboratory computer, installing mouse drivers. Discussions with Mr Fatih Turnalar on duties of the laboratory manager.
25.06.95	am/pm	Return to UK via Istanbul

**Annex 3: Standard Operating Procedures (SOPs)****Annex 3A).****SOP 1: SAFETY IN THE AFLATOXIN LABORATORY.**

1. Eating and drinking should not be allowed in the laboratory.  
[Aflatoxin is a dangerous carcinogen and acute toxin and care must be taken to avoid accidentally ingesting aflatoxin.]
2. Nitrile gloves should be worn at all times, most especially when handling benzene, chloroform and aflatoxin standards.  
[Aflatoxin can be absorbed through the skin, especially when in a benzene solution.]
3. Safety spectacles should be worn at all times, but most especially when handling standards and solvents. Special care is needed when using separating funnels and the rotary evaporator.
4. Laboratory coats should be worn at all times to protect body and clothing from possible contamination or damage.
5. Smoking must not be allowed because flammable solvents (eg. methanol, hexane, benzene, toluene) are in use.
6. All work with aflatoxin standards and with benzene, acetonitrile, chloroform, and hexane must be carried out in a fume-cupboard and great care should be taken not to breath in the solvents. TLC plates must be spotted in a fume cupboard with the front sash down, allowing only arms inside the cabinet.
7. Wear nitrile gloves when holding TLC plates under the UV light.  
[the UV cabinets are fitted with filters (do not clean with solvent, only water) to protect the eyes and the long-wave light used is not as dangerous as short-wave ultra-violet light].
8. Wear dust masks when handling or milling samples. Pistachio nuts and kernels do not create much dust, but it is a sensible precaution.

9. To decontaminate any spillage of aflatoxin standard or extract on skin: immediately soak up the liquid using a tissue. Wash the effected area thoroughly with soap and water. **DO NOT WASH SKIN WITH SOLVENT.**
10. If any solvent splashes in an eye (should not happen if safety spectacles are worn at all times) then wash copiously with a sterile eye-wash (NEED).
11. To decontaminate glass-ware: rinse x 3 with acetone and then wash-up as normal.
12. To decontaminate a spillage on the bench or floor: soak-up with tissue and then wash (wear strong rubber gloves) with a concentrated bleach solution (sodium hypochlorite) and finally rinse with water.
13. To minimise spillages, especially of standards, all vials should be placed in racks and work should be carried out in a shallow containment tray (NEED).
14. Aflatoxin contaminated solutions must be either evaporated to dryness and the glassware decontaminated as in 9). above (use a short soak in bleach if heavily contaminated) or the contaminated solutions can be poured into a remote soil soak-away, away from water supplies, if this is considered safe.

Annex 3B).**SOP 2: METHOD FOR DETERMINATION OF AFLATOXIN STANDARDS AND PREPARATION OF SPIKING SOLUTIONS.**INSTRUCTIONS.

*Whilst using mycotoxin thin films and solutions, the utmost care must be exercised to avoid any personal contact with them. Laboratory coats, nitrile gloves, and safety spectacles must be used at all stages of handling and determination. All work involving mycotoxin standards and toxic solvents, particularly benzene and acetonitrile, must be performed in an efficient fume cupboard.*

To minimise errors it is very important to conduct all operations in suitably subdued natural light using tungsten lighting. Working under fluorescent lighting must be avoided. Standards must be stored in a freezer and when needed they must be thawed in a dark cupboard at room temperature for as short a period as is practical before use. These precautions are needed to prevent degradation of light sensitive toxins, particularly aflatoxins B<sub>1</sub> and G<sub>1</sub>.

PREPARATION OF UV AND STOCK SOLUTION.

1. Remove the vials containing thin films of aflatoxin standard from the freezer and place them in a dark place. Allow them to reach room temperature, over a period of about 1 hour, or accelerate this by warming in a GLOVED hand.
2. Prepare about 500 ml 98:2 v/v benzene / acetonitrile by transferring 490 ml benzene to a 500 ml Erlenmeyer flask and adding 10 ml of acetonitrile. Stopper and store in a fume cupboard.
3. When the standards have attained room temperature, remove the metal cap from the vial to expose the septum. Note the weight of toxin nominally supplied and the LOT number.
4. Insert a syringe needle about 5 mm (not far) through the septum near an edge and leave in place to act as an air vent. [Note: if the air-vent needle is inserted too far then it's end will become submerged when 5 ml solvent is added]

5. Using a 1 ml Tuberculin syringe fitted with a 1.5 inch long needle, add 5 ml benzene / acetonitrile 98:2 for each milligramme of toxin present.
6. Remove the syringe needle acting as an air vent and dissolve the toxin completely using a vortex mixer for at least 2 minutes, or shaking frequently in a gloved hand over a 10 minute period. [The concentration of this stock solution of standard will be approximately 200  $\mu\text{g}$  / ml.]
7. Replace the air vent needle being careful not to submerge it in solvent and, using a clean syringe fitted with a long needle, transfer approximately 0.5 ml of the stock solution to a clean, dry, suitably labelled, 10 ml volumetric flask and make up to the mark with benzene / acetonitrile. Mix well by shaking, and cover the flask with tissue or aluminium foil to protect from light. If the concentration is to be determined within the next 2 hours, then store temporarily in a dark cupboard, otherwise transfer the standard to vials and store in a freezer.
8. Repeat stages 3 to 7 for each of the remaining three toxins. These solutions are known as the UV solutions and have an aflatoxin concentration of about 10  $\mu\text{g}/\text{ml}$ .
9. Store the original aflatoxin standard vials, still fitted with serum caps and containing the residual concentrated aflatoxin solution, in a freezer. These are known as the stock solutions and have an aflatoxin concentration of about 200  $\mu\text{g}/\text{ml}$ .

#### B) CALIBRATION OF THE SPECTROPHOTOMETER.

Procedure for single beam spectrophotometer.

[This dichromate procedure may be omitted if performance of the spectrophotometer can be assessed by means of a certified filter or if it has adequate internal calibration. In these cases, if the spectrophotometer passes the quality assurance test, the correction factor is taken as unity and the analyst can proceed directly to Section C).]

10. Dry about 2-3 g of AR grade potassium dichromate at 150-200°C in an oven for two hours. Store the dried material in a desiccator.

11. Prepare 2 litres of 0.18N sulphuric acid solution by dissolving and mixing 1.00 ml (or 1.84 g) of the concentrated acid in 2000 ml of distilled water.
12. Weigh out about 74 mg of the dichromate **accurately**. Quantitatively transfer it to a 1 litre volumetric flask and make up to the mark using the sulphuric acid solution. Mix well. This solution is about 0.25 millimolar.
13. Pipette 25.0 ml of this solution into a clean, dry 50 ml volumetric flask. Make up to the mark with the 0.18N acid and mix well. This solution is about 0.125 millimolar.
14. Similarly prepare a 0.0625 millimolar dichromate solution by diluting 25.0 ml of the 0.125 millimolar dichromate to 50.0 ml in a clean volumetric flask using the 0.18N acid.
15. Turn on the UV spectrophotometer and let it warm-up for 20 minutes before use.
16. First set the spectrophotometer to the ABS mode. Fill a clean quartz cell with the 0.18N sulphuric acid using a clean Pasteur pipette and put it into the cell holder. The light path is from front to back.
17. Set the wavelength to 350 nm using WAVELENGTH + or -, set to zero using SET REF.
18. Adjust the wavelength to 345 nm. Do not alter any setting other than the wavelength. In 1 nm steps, measure the absorbance at each wavelength, from 345 to 355 nm.
19. Repeat each measurement and calculate the mean.
20. Keep this quartz cell of reference solvent for occasional subsequent checks of the zero reading. Use SET REF to re-set if the reading is 0.002 or more in error.
21. Fill another quartz cell with the most dilute dichromate solution and place it in the cell holder.
22. Other than the wavelength do not alter any setting. Measure the absorbance of this solution as for the solvent alone (see 9 to 10 above) at all wavelengths from 345 to 355 nm.

23. Using a Pasteur pipette, empty the dilute dichromate from the quartz cell. Using a clear pipette, thoroughly rinse the quartz cell with the 0.125 millimolar dichromate x 3 and then fill it.

24. As before, measure the absorbance for a range of wavelengths from 345 to 355 nm in duplicate.

25. Replace and measure the remaining dichromate solution as above.

26. For each of the solutions, subtract the absorbance of the solvent alone at the equivalent wavelength. The value of signs must be taken into account.

27. Find the wavelength at which the absorbance is maximum for each of the three solutions. In each case, the maximum should occur at 350 nm.

28. Calculate the concentration of the most concentrated dichromate solution by dividing the weight taken in milligrammes by the molecular weight (294.18). The concentration is in millimoles per litre. The other dichromate solutions are 1/2 and 1/4 of this concentration.

29. Calculate the molar absorbance,  $E$  at each of the maximum absorption values. ( $E = A/c$ , where  $A$  is the measured absorbance and  $c$  the dichromate concentration in moles per litre).

30. Calculate the mean,  $m$ , of these three values and the coefficient of variation, which should not exceed 0.5 per cent.

31. Calculate the correction factor,  $CF$  for the instrument. This is  $3160/m$ .  $CF$  should always be within the range 0.95 to 1.05. Action is required if this is exceeded.

### C) DETERMINATION OF THE TOXIN CONCENTRATIONS

Note: If a Novaspec II (Pharmacia) single beam spectrophotometer is available then use the 'peak check' mode of operation to automatically determine the absorption at the peak maximum wavelength, go to para 49.

32. Fill a clean dry quartz cell with acetonitrile/ benzene (98:2 v/v), and insert it in the cell holder.



33. Adjust the wavelength to 350 nm and press SET REFERENCE to set up the zero.

*Normal Absorbance Mode*

34. Measure and record absorbance values for the solvent from 340 to 360 nm at 1 nm frequency, as was done for the dichromate solutions.

35. Duplicate the measurements and find the mean.

36. Keep the cell of solvent available for occasional zero checks at 350 nm.

37. Make no adjustment to the spectrophotometer other than for wavelength.

38. Rinse a clean, dry quartz cell with the UV solution for aflatoxin B<sub>1</sub> x 1 and then fill it.

39. Adjust the wavelength to 340 nm.

40. Measure and record the absorbance at wavelengths covering the range from 340 to 360 nm.

41. Duplicate the measurements using a fresh charge of aflatoxin B<sub>1</sub> solution and calculate the mean for each wavelength. Record the absorbance maximum and associated wavelength.

42. Using a clean Pasteur pipette empty the contents of the quartz cell back into the B<sub>1</sub> UV solution.

43. Wash and rinse the cell x 3 with benzene:acetonitrile. Dry the cell with a jet of nitrogen (or air).

44. Rinse the quartz cell x 1 with aflatoxin B<sub>2</sub> UV solution and repeat the procedure from 39 to 43 above.

45. Using a clean Pasteur pipette empty the contents of the quartz cell back into the B<sub>2</sub> UV solution.

46. Repeat the above procedures (steps 39 to 43) for aflatoxins G<sub>1</sub> and G<sub>2</sub>.

47. For each toxin, calculate the true absorbance at each wavelength by subtracting the value for the solvent alone at the same wavelength. Take into account the values of signs.

48. Find the wavelength at which the value is maximum. For the B toxins the maximum value should be around 350 nm: for the G toxins, it should be at 356-357 nm.

#### *Alternative Peak Check Mode Absorbance Measurement*

If available, as on the Novaspec II, this is the preferred procedure because it is very much faster, saving at least 1 hour.

49. Place a clean quartz cell filled with benzene:acetonitrile 98:2 into the spectrophotometer light path. Set the wavelength to 350 nm and press `Set Reference`. The Absorbance should now read 0.000.

50. Measure and store the reference readings +/- 10 nm from 350 nm by pressing simultaneously the `Wavelength +` and `-` keys and holding them down until the display reads `S\_\_b`. Wait for about 3 minutes until the procedure has finished and the display returns to read 350 nm.

51. Fill the cell with aflatoxin standard, as described above, and place the cell in the light path. Press the wavelength + and - buttons simultaneously, but only for a short time until the display reads `Scn`. Wait until the display shows the wavelength of the peak maxima and the associated absorbance.

52. Record these results and repeat the procedure for the other aflatoxins, washing out the cell as described above.

53. The aflatoxin concentration  $c$ , in  $\mu\text{g/ml}$ , is calculated from the following formula:

$$c = \frac{\text{ABS} \times \text{Mwt} \times \text{CF} \times 1000}{E \times L},$$

where ABS is the maximum absorbance recorded for the particular toxin, Mwt its molecular weight, E its molar absorbance and L the cell path length, (in this case  $L = 1$ ). The molecular weights and molar absorptivities are respectively:

B<sub>1</sub>, 312 and 19,800,  
 B<sub>2</sub>, 314 and 20,900,  
 G<sub>1</sub>, 328 and 17,800  
 G<sub>2</sub>, 330 and 18,200.

54. Use the attached form (QA-010) to record results and calculate the concentration of the standards.

D) MAKING UP STANDARDS AND SPIKING SOLUTIONS

55. Transfer, using a Hamilton syringe, 0.5 ml of B<sub>1</sub> and G<sub>1</sub> UV solutions and 0.25 ml of B<sub>2</sub> and G<sub>2</sub> UV solutions into a clean, dry 10 ml volumetric flask (or use other volumes to effect a x20 dilution).

56. Make up to the mark with benzene/acetonitrile (98:2 v/v). Mix well by shaking the volumetric flask.

57. The concentration of the aflatoxins in µg/ml is:

$$\frac{V \times C}{F}$$

where V is the volume of UV solution used in ml and C is the concentration of the UV solution in µg/ml and F is the volume of the volumetric flask used for the dilution. This mixed standard solution contains about 0.5 µg/ml for B<sub>1</sub> and G<sub>1</sub> and 0.3 µg/ml for B<sub>2</sub> and G<sub>2</sub>.

58. Transfer about 2 ml of the mixed aflatoxin standard into several clean 3.5 ml tall form, narrow-necked vials. Stopper tightly and cover with Parafilm to minimise losses due to evaporation of solvent.

59. Each vial should be labelled with its date of preparation, concentration of the aflatoxins and the batch number so that it can be used in strict rotation.

60. Store each vial in a freezer when not in use.

61. These vials contain a volume of standard sufficient to minimise the effects of evaporation while spotting.

62. Use each standard for spotting a maximum of five plates. The next plate spotted should compare the old standard with the new standard. Differences of more than 5 per cent are

not acceptable and reasons for them should be tracked down and eliminated.

63. The UV solutions of the individual toxins can be used directly for spiking, but care must be taken that the solvent used for the standard and that used in the extract to be spiked are completely miscible. If this is not the case, then take the required volume of standard down to dryness, preferably under nitrogen, and dissolve directly in aliquots of the extract.

**Annex 3C****SOP. SEEM-QUANTITATIVE AND QUANTITATIVE TLC DETERMINATION  
OF AFLATOXIN IN PISTACHIOS AND RED PEPPERS (Sterner Method  
based on BOAC, 'B.F.' METHOD)**

## Sources:

I. Sterner W.E. et al. *J. Agric. Food Chem.* 1992 **40**, 2453-2457

II. *Official Methods of Analysis of the AOAC*, Published by Association of Official Analytical Chemists, Washington, D.C., 15th Ed. 1990. 'BF' Method, Section 970.45, 1190-1191.

The AOAC 'BF' method has been officially adopted for the analysis of aflatoxin in groundnuts and groundnut products and has been found applicable to pistachios with the modifications made by Steiner. It may also prove suitable for the analysis of red pepper and other commodities produced in South Eastern Anatolia.

**TLC ANALYSIS OUTLINE****EXTRACTION**

200 ml METHANOL + (20+60) WATER

**CLEAN-UP**

PARTITION WITH HEXANE

PARTITION INTO CHLOROFORM

**WORK-UP**

EVAPORATE TO DRYNESS

TRANSFER TO VIAL

DETECTION by TLC (>1 ppb)

SEMI-QUANTITATIVE TLC

QUANTITATIVE TLC

**METHOD**

Follow the flow-chart after reading these notes.

1. Carry out the work in a laboratory which is shielded from direct sun-light and use only tungsten artificial lighting. Do not use fluorescent lighting.
2. A fume-hood is essential for plate spotting using benzene:acetonitrile dilution solvent.
3. Aflatoxin working standards must be kept in a freezer when not in use and great care must be taken to prevent evaporation of solvent during use. Each vial of standard should be used to spot no more than 5 quantitative plates. It can then be used as a qualitative standard.
4. See Figure 1 for spotting patterns for TLC plates to be used for: detection, semi-quantitative, and quantitative analysis.
5. A list of the reagents and apparatus required is given in Appendix 1 to this SOP.

**FLOW CHART****TLC METHODS FOR AFLATOXIN ANALYSIS OF PISTACHIO NUTS****A. Pistachio Kernels using Paste**

<b>MILLING</b>	
1).	Kernels 10 kg kernels in Vertical Cutter/Mixer fitted with Cutter Blade.
2).	Run 2 x 30 seconds at speed II Remove 5 kg semi-paste Run 30 seconds at speed II with remaining 5 kg

**EXTRACTION**

- 3). Weigh out 80 g paste into a 1 litre blender jar
- 4). Add 200 ml methanol + 20 ml water
- 5). Blend at high speed for 2 minutes
- 6). Add 60 ml water
- 7). Blend for further 1 minute
- 8). Filter through Whatman No. 2 filter paper

**CLEAN-UP**

- 9). Measure 70 ml filtrate into a 500 ml separating funnel
- 10). Add 50 ml hexane (to remove lipids)
- 11). Add 55 ml water
- 12). Add 2 g sodium chloride (to prevent emulsions), ensuring that none becomes attached to the socket for the stopper, otherwise it will leak
- 13). SHAKE funnel for 1 minute, releasing pressure frequently by removing the stopper
- 14). Allow layers to separate
- 15). Run off lower layer into second separating funnel
- 16). Add 25 ml chloroform
- 17). SHAKE funnel for 30 seconds., releasing pressure as

in 13). above

- 18). Allow layers to separate
- 19). Run off lower layer into 250 ml conical flask containing 2 g anhydrous sodium sulphate.
- 20). Repeat steps 16-19 with second 25 ml chloroform
- 21). Decant combined extract into 250 ml round bottomed flask., being careful not to transfer sodium sulphate
- 22). Take to dryness on a rotary evaporator at 45 °C
- 23). Transfer to 7 ml vial using 3 x 1 ml chloroform
- 24). Take to dryness at 45 °C under jet of air  
(Note: nitrogen gas preferred to air)

### SEMI-QUANTITATIVE TLC

From step 22 or 24).

- 25). Add 1 ml benzene:acetonitrile 98:2 to the dry extract
- 26). Vortex mix, 30 seconds
- 27). Spot 5  $\mu$ l spots in duplicate on TLC plate  
(if at step 32). the 5  $\mu$ l spot is visible, then > 1 ppb)  
  
Go to step 31). for 1 ppb Screening Plate  
  
For semi-quantitative range > 1 < 4 > 4 < 10 > 10
- 28). Add extra 3 ml benzene:acetonitrile to the extract
- 29). Vortex mix 30 seconds
- 30). Spot 2 and 5  $\mu$ l spots in duplicate on TLC plate
- 31). Develop plate for 15 minutes in chloroform:acetone:  
water 88:12:0.2



- 32). Dry plate for 5 minutes in a fume hood and observe under long-wave UV light
- D = 1 ml: If 5  $\mu$ l spot not visible, then < 1 ppb aflatoxin  
If 5  $\mu$ l spot just visible, then > 1 ppb aflatoxin
- D = 4 ml: If 5  $\mu$ l spot not visible, then < 4 ppb  
If 5  $\mu$ l spot visible, then > 4 ppb  
If 2  $\mu$ l spot not visible, then < 10 ppb  
If 2  $\mu$ l spot visible, then > 10 ppb aflatoxin

If > 1 ppb aflatoxin

### QUANTITATIVE TLC

- 33). Take extract to dryness under stream of nitrogen (air) in water bath at 45°C.
- 34). Add 500  $\mu$ l benzene:acetonitrile and vortex mix 30 sec.
- 35). Spot HPTLC plate with 1, 2, 3, 4, and 5  $\mu$ l of sample and working standard solutions.
- 36). Develop plate for 15 minutes in chloroform:acetone:water 88:12:0.2
- 37). Dry plate and observe under long-wave UV
- 38). Compare intensity of spots under long-wave UV light and record best equivalence.
- Y  $\mu$ l sample - Z  $\mu$ l standard
- 39). Calculate result in  $\mu$ g/kg (ppb) using equation:  
ppb =

$$\frac{C \times \text{Volume of standard spot (Z)} \times D}{\text{Volume sample spot (Y)} \times \text{EWt}}$$

Where C = concentration of standard in  $\mu$ g/ml

D = Dilution in  $\mu$ l (= 500)

EWt = Effective weight in final extract (= 20 g)

**B. Pistachio Nuts in Shell using Paste**

<b>MILLING</b>	
1).	5 kg whole nuts in Vertical Cutter/Mixer fitted with Mixing Insert
2).	Run 30 seconds at speed II
3).	Repeat with second 5 kg for 10 kg sample
4).	Sieve through 2 mm screen to separate kernels from shell.
5).	Put kernels back into Vertical Cutter/Mixer fitted with Cutter Blade
6).	Run 30 seconds at speed II (for paste)

Proceed as for Method A for pistachio kernels, Step 3).

**REAGENTS AND APPARATUS****Reagents**

Chloroform A.R.

Acetone A.R.

Hexane.

Methanol A.R.

Sodium chloride.

Sodium sulphate, anhydrous

(Nitrogen).

**Apparatus**

Blender, 1 litre.

Measuring cylinders, 10, 100, 250 ml.

Pipettes, 2 ml,

Pasteur pipettes and rubber bulbs

Separating funnels, 500 ml.

Beakers, 100 ml.

Vials, 7 ml squat.

Diaphragm pump

Rotary vacuum evaporator with water bath.

UV light, 365 nm, preferably in a UV viewing cabinet.

HPTLC precoated plates, silica, aluminium-backed 20x20 cm to be cut to 20x10, without fluorescent indicator, Merck 5547 (or glass-backed). Best for quantitative TLC.

TLC pre-coated plates, silica, aluminium-backed 20x20 cm to be cut to 20x10 cm, without fluorescent indicator, Merck 5553, (or glass-backed). For detection and semi-quantitative TLC, but quantitative TLC is also possible.

TLC tank, 20x20 with good fitting lid of glass or metal, preferable with vee-trough in base

Micro-cap capillaries for plate spotting, 1, 2, and 5  $\mu$ l.

Pencil, 2B and wooden ruler for marking out TLC plates

## TLC SPOTTING PATTERNS

## TLC Spotting Patterns

Figure 2. Spotting Pattern for TLC Screening Plate to Detect > 1 ppb  
Dilution of extract = 1 ml benzene:acetonitrile 98:2

Sample	A	B	C	D	E	F	G	St	H	I	J	K	L	M	N	O
	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Volume	5 $\mu$ l	5 $\mu$ l	5 $\mu$ l	5 $\mu$ l	5 $\mu$ l	5 $\mu$ l	5 $\mu$ l	2 $\mu$ l	5 $\mu$ l	5 $\mu$ l	5 $\mu$ l	5 $\mu$ l	5 $\mu$ l	5 $\mu$ l	5 $\mu$ l	5 $\mu$ l

St. = Qualitative aflatoxin B1 standard

Assumes that the minimum weight of aflatoxin that can be just seen on a TLC plate is 0. Therefore if 5  $\mu$ l can be seen it must contain at least 0.1 ng.

Therefore 1000  $\mu$ l extract must contain at least  $1000 \times 0.1 / 5 \text{ ng} = 20 \text{ ng} / 20 \text{ g} = 1 \text{ ng/g}$   
1 ppb

Figure 3. Spotting Pattern for TLC Semi-Quantitative Plate

Dilution of extract = 4 ml benzene:acetonitrile 98:2

Sample	A	A	A	A	B	B	B	B	St	C	C	C	C	D	D	D	D
	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Volume	2 $\mu$ l	2 $\mu$ l	5 $\mu$ l	5 $\mu$ l	2 $\mu$ l	2 $\mu$ l	5 $\mu$ l	5 $\mu$ l	2 $\mu$ l	2 $\mu$ l	2 $\mu$ l	5 $\mu$ l	5 $\mu$ l	2 $\mu$ l	2 $\mu$ l	5 $\mu$ l	5 $\mu$ l

St = Qualitative aflatoxin standard

If the 2  $\mu$ l spot can be seen then > 10 ppb, if not visible then < 10 ppb

If the 5  $\mu$ l spot can be seen then > 4 ppb, if not visible then < 4 ppb

Figure 4. Spotting Pattern for Semi-Quantitative Plate, no previous 1 ppb Screen.

	Dil. =	Dil. =	Dil. =		Dil. =	Dil. =	Dil. =		Dil. =	Dil. =	Dil. =		
	1	4	4		1	4	4		1	4	4		
Sample	A	A	A	A	A	A	St	B	B	B	B	B	
	+	+	+	+	+	+	+	+	+	+	+	+	
Volume	5 $\mu$ l	5 $\mu$ l	2 $\mu$ l	2 $\mu$ l	5 $\mu$ l	5 $\mu$ l	2 $\mu$ l	5 $\mu$ l	5 $\mu$ l	2 $\mu$ l	2 $\mu$ l	5 $\mu$ l	5 $\mu$ l

St. = Qualitative aflatoxin B1 standard  
 Dil. = Dilution of sample in Bz:ACN 98:2

If 5  $\mu$ l Dilution = 1 can be seen, then > 1 ppb  
 If 5  $\mu$ l Dilution = 4 can be seen, then > 4 ppb  
 If 2  $\mu$ l Dilution = 4 can be seen, then > 10 ppb

Figure 5. Spotting Pattern for TLC Quantitative Plate

Dilution of sample extract = 500  $\mu$ l

	HPTLC Plate														
Sample	A	A	A	A	A	St	St	St	St	St	B	B	B	B	B
	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Volume	1 $\mu$ l	2 $\mu$ l	3 $\mu$ l	4 $\mu$ l	5 $\mu$ l	1 $\mu$ l	2 $\mu$ l	3 $\mu$ l	4 $\mu$ l	5 $\mu$ l	1 $\mu$ l	2 $\mu$ l	3 $\mu$ l	4 $\mu$ l	5 $\mu$ l

St = Quantitative mixed aflatoxin standard

**'STEINER' TLC METHOD: ANALYSIS OUTLINE**

**EXTRACTION**

**80:20 METHANOL WATER**

**CLEAN-UP**

**PARTITION WITH HEXANE**

**PARTITION INTO CHLOROFORM**

**WORK-UP**

**EVAPORATE TO DRYNESS**

**TRANSFER TO VIAL**

**DETECTION by TLC (>1 ppb)**

**SEMI-QUANTITATIVE TLC**

**QUANTITATIVE TLC**

## Annex 3D

## SOP 4: CONFIRMATION AND QUALITY ASSURANCE.

## A. CONFIRMATION OF AFLATOXIN

1. It is very important to confirm that the fluorescent spots that are quantified really contain aflatoxin. To do this a hemi-acetal derivative is formed on the HPTLC plate prior to development. Aflatoxins B<sub>1</sub> and G<sub>1</sub> form derivatives which run slower than the parent aflatoxins. If these aflatoxins really are present then the parent spots disappear, or become far less intense, and a new slower running spot corresponding to the hemi-acetal derivative appears. Aflatoxins B<sub>2</sub> and G<sub>2</sub> do not form derivatives and therefore can not be confirmed using this method.

2. At least 5% of all positive samples analysed should be routinely confirmed by the method given below.

(a) Use a spotting pattern which allows up to 5 sample extracts and one spot each of standard aflatoxin B<sub>1</sub> and G<sub>1</sub> to be spotted on both the left and right side of an HPTLC plate. Leave a space of at least 2.5 cm between the two sides of the plate. Mark the base-line and track positions at 10 mm intervals with a pencil before spotting.

(b) After spotting with 5  $\mu$ l of extract and 2  $\mu$ l standard, place the plate in a fume cupboard and cover the left hand side of the plate with a clean plate of glass or a wooden ruler.

(c) On the right hand side of the plate carefully over-spot each track with 1  $\mu$ l of trifluoroacetic acid (TFA).

**CARE.** TFA vapour is harmful to eyes and causes tear formation even at very low concentrations in the air.

(d) Allow the plate to stand in a darkened fume cupboard for 5 minutes to allow the derivatisation reaction to complete and to evaporate excess TFA.

(e) Develop the plate with chloroform:acetone:water (CAW) 88:12:0.2 for 15 minutes.

(f) Examine the plate and check that the standards on the right hand side of the plate have derivatised. If they have, check the tracks spotted with sample extracts. All samples on the right hand side of the plate which have lost the suspected aflatoxin B<sub>1</sub> and G<sub>1</sub> spots and have gained a spot corresponding to the hemiacetal derivative are confirmed.

**Figure 6. Spotting Pattern for TLC Confirmatory Test using TFA**

Dilution of extract = 500  $\mu$ l benzene:acetonitrile 98:2

		TLC Plate																						
		No TFA added						TFA over-spotted																
		0	0	0	0	0	0							0							0			
														0	0	0		0						
							St.							St.										
Sample		A	B	C	D	E	B1							B1	A	B	C	D	E					
		+	+	+	+	+	+							+	+	+	+	+	+					
Volume		5 $\mu$ l	5 $\mu$ l	5 $\mu$ l	5 $\mu$ l	5 $\mu$ l	2 $\mu$ l							2 $\mu$ l	5 $\mu$ l	5 $\mu$ l	5 $\mu$ l	5 $\mu$ l	5 $\mu$ l					
	Trifluoroacetic acid to form hemiacetal derivative ---->													1	1	1	1	1	1					
														$\mu$ l	$\mu$ l	$\mu$ l	$\mu$ l	$\mu$ l	$\mu$ l					

St = Qualitative aflatoxin standard

To confirm aflatoxins B<sub>1</sub> and G<sub>1</sub>:

If the suspect aflatoxin spot in the sample behaves the same as the standard when over-spotted with TFA ie.

- i). original spot disappears
- ii). new slow-running blue hemiacetal derivative spot appears at same R<sub>f</sub> as standard

then suspect spot is probably not aflatoxin.

If the suspect aflatoxin spot does not behave the same as the standard when over-spotted with TFA, then it is not aflatoxin



**QUALITY ASSURANCE**

3. There is a danger that even when SOPs are rigorously adhered to that some systematic error may creep in. This may result in a bias towards high or low results and it is essential to detect this as quickly as possible. Failure to do so will result in unreliable data and misleading conclusions.

a. Select a recently analysed, aflatoxin-positive (>10 ppb), **AND** an aflatoxin negative finely ground sample (or paste) of a commodity under study.

b. Weigh out 12 x 100g aliquots of the well mixed sample

c. Place the sub-samples into small plastic bags and seal. Label each bag to show that it is a quality assurance sample and state the commodity, whether it is aflatoxin positive or negative and the date. Store in a freezer.

d. Take two of the analytical aflatoxin-positive samples and weigh out 80 g of each. Analyse for aflatoxin by the routine procedure (If no aflatoxin positive sample is available move to (h))

e. Record the results on a Quality Assurance spreadsheet. The mean value is taken as the baseline aflatoxin level.

f. Analyse further quality assurance samples MONTHLY, or more frequently if a systematic error is suspected.

g. Plot the monthly results looking for any upward or downward trends. If the result deviates by more than 5% from the previous determination, or by more than 10% from the original baseline level, then repeat the analysis and if the result is confirmed troubleshoot to try to determine the reason.

h. Check the clean-up and quantification steps by carrying out a recovery experiment on aflatoxin-spiked extract of the aflatoxin-negative quality assurance sample. If recovery is less than 90%, then look for any changes in: procedure, origin or grade of solvents and reagents, concentration of standards (low results) or

degradation of standards (high results, or environmental changes which might account for the losses.

4. If no error is identified, note any discrepancy and resume routine analysis and quality assurance. Take any discrepancy into account when interpreting the results.

5. Each spotting standard should be used for a maximum of five (5) plates. On the fifth plate both old and new standards should be spotted to check for consistency.

Date SOP 4 Prepared: 15 June 1995.

Author: Martin J. Nagler, UNIDO Consultant

**Amendments:**

**Amendment 1.**

Date:

Paragraph (s) amended:

Reason:

Validation:

Authorised by:

Copy paragraphs to be replaced here:

Type Amendments in SOP above.

**ANNEX 4: Training Course Programme and Text of Training  
Course Lectures**

**Annex 4A). Training Course Programme**

**UNIDO AFLATOXIN TRAINING COURSE: QUALITY CONTROL  
FOR PISTACHIO NUTS**

Venue: UNIDO/GTS Aflatoxin Laboratory

Date: 21-23 June 1995

**21.06.95**

9.00-9.30 Registration

9.30-10.00 Introductions

Welcome (Mr Hasan Ozturkmen, President GTS)

Trainees Introduce themselves

Project Background/Aims of the Course  
(Prof. Yildiz)

Aflatoxin and its Significance (Prof Öner)

10.00-10.30 Methods of Aflatoxin Analysis: Principles  
and Criteria for Selection

10.30-10.45 Break

10.45-11.15 Talk: Sample Collection and Sample  
Preparation, Nature of aflatoxin distribution  
and practical solutions.

11.15-12.00 Safety in the Laboratory

12.00-12.30 Demonstration, milling of pistachio nut and  
pistachio kernels using vertical cutter  
mill.

12.30-13.30 Lunch

13.30-14.00 Talk: Steiner TLC Method, Flow Diagram

- 14.00-15.00 Demonstrations of Steiner TLC qualitative aflatoxin screening method: extraction/clean-up and work-up.
- 15.00-15.15 Break
- 15.15-15.30 Demonstration of TLC plate preparation and spotting
- 15.30-15.45 Talk: Principles of TLC Semi-Quantitative Screening Method
- 15.45-17.00 Calibration of trainees minimum level of aflatoxin detection on a TLC plate  
Reading a Semi-Quantitative TLC plate
- 22.06.95**
- 09.30-10.00 Talk: Preparation, Maintenance, and Use of Aflatoxin Standards
- 10.00-10.30 Talk: Principles of Quantitative TLC and Calculation of Results
- 10.30-10.45 Break
- 10.45-12.30 Demonstration of spotting a quantitative TLC plate and developing it.  
Demonstration and practice of plate reading
- 12.30-13.30 Lunch Break
- 13.30-13.45 Talk: Confirmatory Tests and Quality Assurance
- 13.45-15.00 Demonstration of confirmatory test and interpretation
- 15.00-15.15 Break
- 15.15-17.00 Hands on plate spotting  
Plate reading exercises  
Plate calculation exercises  
Questions Arising

**23.06.95**

09.30-10.30      Talk: Aflatoxin Kits, general principles,  
                         advantages and disadvantages.  
                         Cortecs Total Aflatoxin Kit Flow-Chart

10.30-10.45      Break

10.45-11.30      Demonstration of Cortecs kit to first  
                         incubation

11.30-12.30      Talk: EZ-Screen plus demonstration

12.30-13.30      Lunch Break

13.30-14.00      Cortecs kit continued

14.00-15.00      Easi-Extract clean-up/Cortecs kit continued

15.00-15.15      Break

15.15-16.00      Easi-Assay Mini-Column

16.00-16.30      Cortecs ELISA kit plate reading/calculation

16.30-17.00      Round-up: Choice of Analytical Methods.

**Annex 4B).****UNIDO AFLATOXIN LABORATORY, Background Objectives & Status.**

## BACKGROUND

The Turkish Government requested UNIDO to assist the Pistachio Processing Sector in May 1990. In response, Dr Kamal Hussein, then the UNIDO Country Director, visited the major pistachio production regions of Gaziantep and Nizip in South Eastern Turkey in July 1990. He consulted widely and then, together with Mr Mesut Ölcal the Secretary General of the Gaziantep Chamber of Commerce, identified aflatoxin contamination as a possible constraint to the expansion of the pistachio industry. Dr Hussein recommended a UNIDO-funded project as a pro-active response to the perceived aflatoxin threat, and this project was approved in July 1992 with a budget of US\$ 98,000.

The UNIDO Country Director, together with the National Project Director Prof. Fatih Yildiz, revisited Gaziantep and Nizip over 7-9 April 1993 to lay the groundwork for project activities, including preparing for the first part of a split mission by a UNIDO mycotoxin consultant.

The UNIDO mycotoxin consultant, Mr Martin Nagler, visited Gaziantep for a two week period commencing 12 July 1993. His report contained an appraisal of the pistachio industry and a recommendation on appropriate aflatoxin quality assurance procedures. He assessed possible locations for a proposed UNIDO aflatoxin laboratory and recommended the analytical methodologies and equipment to be employed.

UNIDO decided to site the aflatoxin laboratory at the Guneydogu Tarim Satis Factory, Gaziantep, following a kind offer by the President, Mr Hasan Öztürkmen, to provide laboratory accommodation, technical staff, and equipment to complement that provided by UNIDO. The Gaziantep Chamber of

Commerce and the University of Gaziantep offered support and a Board of Directors was appointed.

#### OBJECTIVES OF THE LABORATORY

1. To provide aflatoxin quality control for the pistachio sector in South Eastern Anatolia which will become recognised internationally as reliable
2. To extend aflatoxin quality control to other products of the region as required
3. To carry out aflatoxin surveys to monitor and study aflatoxin contamination in susceptible commodities
4. To provide training in aflatoxin quality control procedures

#### STATUS OF THE LABORATORY

The laboratory is fully commissioned and is equipped to carry out aflatoxin quality control to ISO 9000, as an integral part of the GTS factory. It has written standard operating procedures and trained staff. It is planned that results will be recorded on a computer data-base and will be despatched world-wide by fax.

Aflatoxin standards are regularly checked for concentration at the Food Engineering Department, University of Gaziantep, with the kind assistance of Prof. Memut Öner.

The laboratory is equipped with a 25 litre vertical cutter/mixer mill to handle 20 kg bulk samples of pistachio nuts, in-shell or kernels, and produce representative laboratory and analytical samples

Qualitative screening of pistachio samples is by a published thin layer chromatographic (TLC) method which provides rapid, reliable results to the required detection limits. Positive samples are quantified by TLC using visual comparison with standards.

Staff are also trained to use a range of aflatoxin kits, both for rapid screening and for fully quantitative results by enzyme linked immuno-sorbent assay (ELISA).

The UNIDO laboratory will link with reference aflatoxin laboratories in Turkey and Europe to regularly check and confirm its performance, and thus achieve international recognition.

Prepared by M J Nagler  
UNIDO Mycotoxin Consultant  
14 June 1995



**Annex 4C).****AFLATOXIN AND ITS SIGNIFICANCE****WHAT IS AFLATOXIN?**

Aflatoxin is the name given to four closely related, see Figure 1, toxins; aflatoxins B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, and G<sub>2</sub>, which are produced mainly by the moulds *Aspergillus flavus* and *Aspergillus parasiticus*. Toxins produced by moulds are called mycotoxins and currently about 300 such toxins have been discovered. Relatively few moulds produce toxins, and in fact many produce beneficial drugs, such as penicillin.

Aflatoxin was discovered at the Tropical Products Institute, now the Natural Resources Institute, in England, where it was found to have been the cause of 'Turkey X' disease which killed over 100,000 Turkey poults, as well as numerous calves and pigs, in 1960.

Aflatoxin B<sub>1</sub> occurs in the largest quantities and is the most toxic of the aflatoxins. It is the most potent chemical carcinogen known, causing liver cancer in a wide range of test animals. It is also an acute toxin, with low levels causing death in test-animals within 21 days. Aflatoxin can also reduce the effectiveness of the immune system, making animals more susceptible to viral and bacterial diseases.

Epidemiological studies strongly indicate that aflatoxin, together with the hepatitis B virus, is a major cause of human liver cancer.

More than 50 countries now have legislation limiting quantities of aflatoxin in foodstuffs. These limits are very low, usually in the range of 4 to 20 µg/kg (or parts per billion, ppb) total aflatoxin for nut and nut products.

**WHEN DOES AFLATOXIN CONTAMINATION OCCUR?**

Aflatoxin producing moulds infect crops and grow when moisture contents exceed those in equilibrium with a relative humidity in excess of about 80% and when temperatures are in the range 15-55°C, although large quantities of aflatoxin are only produced when temperatures are in the range 25-35°C. *Aspergillus flavus* and *A. parasiticus* appear as yellow-green sporulating heads, which are easy to detect, but with age

they darken through green into brown and become less noticeable and harder to identify. Finally, when conditions for growth have passed, the sporulating heads crumble to dust and there is no longer a visible sign of the mould, but the aflatoxin that it produced remains.

Crops vary widely in their susceptibility to aflatoxin contamination with maize and groundnuts being highly susceptible, pistachios, red pepper and figs moderately susceptible, and soya and rice only slightly susceptible under normal systems of production and post-harvest handling.

For pistachios, aflatoxin contamination can occur when the moisture content of the kernel exceeds about 15%. Pre-harvest contamination has been associated with split-hulls and tattering, which is reported to be rare in Turkey. In Turkey it is thought that storage of pistachios in-hull could minimise aflatoxin contamination of kernels during storage. However, nuts would become more susceptible to contamination after de-hulling by the wet process, when insufficient drying could allow mould growth and aflatoxin production in the kernels. Studies are required to check that this theory is correct.

Prepared by M J Nagler  
UNIDO Consultant  
14 June 1995

**Annex 4D).****METHODS FOR AFLATOXIN ANALYSIS: PRINCIPLES  
AND CRITERIA FOR SELECTION**

There are two main types of assay which have been developed for detection and/or determination of mycotoxins, viz. biological and chemical. Bioassay techniques, are only qualitative or semiquantitative and are often non-specific. They are useful for indicating the presence of unknown toxins, and are useful in studies to isolate new mycotoxins. Once a new toxin has been identified, it is then usually possible to develop a suitable chemical assay for its detection and quantification. The chemical assay is almost invariably quicker, cheaper, more specific, more reproducible, and more sensitive than the corresponding bioassay. Chemical assays are suitable for routine analyses, as required for quality control and survey work. Immunoassays, are a combination of a chemical and a biological assay. They are very sensitive and can be specific, making them suitable for screening body fluids etc., for traces of toxin.

**CHEMICAL ANALYSES**

All methods of chemical analysis for mycotoxins incorporate a combination of procedures, as indicated in the flow-chart in Figure 1.

**Sampling and Sample Preparation**

If the sample taken for analysis is not representative of the bulk, then the analytical results are meaningless. Because of the very uneven distribution of mycotoxins that are usually found in naturally contaminated commodities, it is essential to follow a suitable sampling plan to provide a

representative primary laboratory sample. Sample preparation involves grinding and sample division to obtain a representative analytical sample. Sub-sampling mills exist, such as the NRI mill, can carry out both milling and sample division simultaneously. The ground primary sample should either be free-flowing for subsequent mixing and sample division, or should be a homogeneous paste. Use of water slurries, especially for samples which are not free-flowing, enables larger, and therefore more representative, analytical samples to be taken. Typically, 1 kg of ground material is blended at high speed with an appropriate amount of water to give an homogeneous slurry from which 100 g aliquots are taken for analysis.

### **Extraction**

The organic solvents most commonly used for extraction of mycotoxins are: chloroform, acetonitrile, methanol and acetone. These solvents are mixed with a given ratio of a more polar solvent e.g. water, dilute acid, aqueous solution of salts, to aid the breaking of weak electrostatic bonds which bind some mycotoxins to other substrate molecules, e.g. proteins.

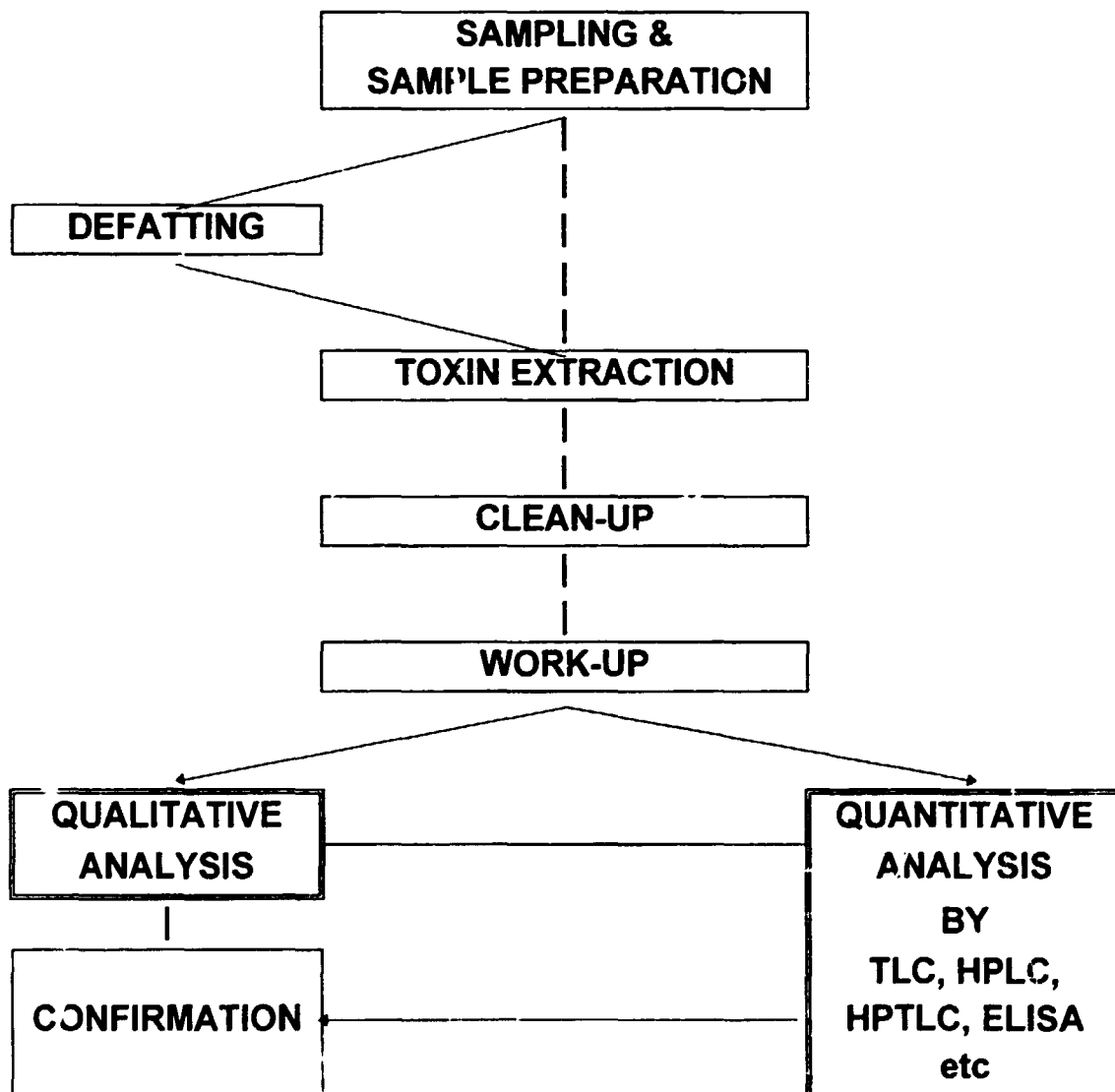
The ground sample, or preferably an aqueous slurry, is either shaken with the extraction solvent for 30-45 minutes or blended at high speed for about 3 minutes. It should be noted that an explosion proof blender is recommended for use with inflammable solvents such as acetone and hexane.

### **Clean-Up**

Mycotoxins are such a diverse group of chemical compounds, that it is difficult to find a simple procedure which specifically removes non-mycotoxin 'interfering' compounds whilst leaving the mycotoxins in the extract. For this

reason it is difficult to find a good method for screening a wide-range of mycotoxins simultaneously. It is possible, however, to devise procedures which remove interfering non-mycotoxin

Figure 7. Flow Diagram for Mycotoxin Analysis



compounds from the extract of a particular commodity and leave a particular mycotoxin or group of mycotoxins in the extract.

Common clean-up techniques which have been used are:

(a) Defatting: this is carried out prior to the toxin extraction step and uses petroleum ether or hexane to extract lipids from the sample using a Soxhlet extractor. This step is only required when the subsequent clean-up step is not capable of removing the lipids.

(b) Column-chromatography: this technique has very wide application, and is used in a number of regulatory or officially approved methods. A glass column is packed with one or more adsorbent materials and the crude extract is added to the top of the column. The column is then eluted with a series of solvents or solvent mixtures which are designed to first wash off interfering compounds and then elute the desired mycotoxins, whilst other interfering compounds remain strongly bound on the column. Traditionally column chromatography was carried out using 10 g of packing material which required hundreds of ml of solvents, but now this can often be scaled down by a factor of twenty, in commercially available columns.

(c) Solid Phase Extraction: is an adaptation of the scaled down columns described above, but the packing material is bonded-phase silica which can improve selectivity. Common bonded phases are C<sub>18</sub> and C<sub>8</sub> reverse phases, phenyl, and quaternary ammonium anion exchange such as SAX. The routine method of HPTLC aflatoxin analysis used at the Natural Resources Institute uses phenyl bonded-phase columns

(d) A miniature glass column, called a mini-column is used in many rapid aflatoxin assay methods to remove interfering compounds and to qualitatively detect aflatoxin down to a few parts per billion (ppb).

(e) Precipitation: this is a useful technique whereby certain chemicals, sometimes in colloidal form, are added to the crude extract and these adsorb certain pigments, proteins and other interfering compounds onto their surface. The complex so formed precipitates out of solution and can be filtered off, leaving a "cleaned-up" solution. Useful precipitating agents include: cupric carbonate, ammonium sulphate, lead acetate, and ferric gel.

(f) Liquid-liquid partition: is commonly used, often in conjunction with one of the other clean-up procedures, to provide additional clean-up and also to transfer toxins from one solvent system to another whilst at the same time effecting a considerable increase in concentration of the toxins. The partition is carried out in a separating funnel which contains the two immiscible solvents. The funnel is shaken for a few minutes to allow the dissolved compounds, including the mycotoxins, to partition between the two phases. The solvents are selected so that the mycotoxins are preferentially partitioned into one of the solvents. Care must be taken in the choice of solvents and use of ionic additives, such as salt, in order to minimise the risk of emulsion formation.

(g) immuno-affinity columns: these are columns containing bound anti-bodies which are specific to particular toxins or families of toxins. Crude extract is diluted with buffer, to reduce the proportion of the organic component, and the toxin is selectively removed. After washing the column, the toxin is eluted with a solvent capable of breaking the antibody-toxin linkage.

This type of clean-up is often used in conjunction with HPLC, but it can also be used with HPTLC or mini-column, after a solvent change is effected

(h) Thin layer chromatography (TLC): although this technique is used mainly for detection and quantification of toxins it is also useful for separating the mycotoxins from interfering compounds in the extract. Sometimes it is necessary to try a range of developing solvents in order to separate the toxins of interest from the interfering compounds. If this proves impossible, or too time consuming, then two-dimensional TLC or bi-directional TLC may be applicable.

(i) Other clean-up procedures: The clean-up procedures outlined above are those employed in officially approved methods for aflatoxin analysis. Other clean-up procedures include reverse dialysis and gel electrophoresis for multi-mycotoxin screens and base extraction which is useful for extracting acidic or phenolic mycotoxins, such as ochratoxin and zearalenone.

### **Work-Up**

After the clean-up step the extract must be 'worked-up' in order to prepare it for the detection and/or quantification step. Following clean-up the extract is often dissolved in a large volume of aqueous solvent, so it must be transferred into a small volume (10-50 ml) of a volatile solvent, such as chloroform. The chloroform solution often requires drying and this is achieved by passing it through a bed of anhydrous sodium sulphate. The solvent is evaporated off, as described below, to near dryness. Care must be taken at this stage as some mycotoxins, e.g. the aflatoxins, can break-down if the dry extract is heated at 100°C. To avoid this the evaporation is best carried out using a rotary evaporator at 45°C to reduce the volume to a few ml, followed by



evaporation to dryness in an inert atmosphere using a sample concentrator. If such items of equipment are not available, then evaporation should be carried out using a steam-bath, preferably under a stream of nitrogen, and great care should be taken to ensure that flasks are removed from the steam-bath just before they go dry, and that vials are removed on the point of dryness.

The extracts are now ready for detection and quantification.

### **Detection and Quantification**

It is fortunate that the aflatoxins and many of the other important mycotoxins are fluorescent under ultra-violet light. This enables them to be detected at very low levels ( $\mu\text{g}/\text{kg}$  or parts per billion, ppb), and this method of detection is used in the majority of analytical methods for mycotoxins.

Detection or 'qualitative assay' is usually by TLC, mini-column, or kit, employing enzyme linked immuno-sorbent assay (ELISA) or immuno-affinity. These methods either do not require a standard, or only requires the use of a qualitative (not accurately determined) standard. Such a standard is also sufficient to enable a semi-quantitative assessment to be made by TLC, using a 'dilution-to-extinction' principle.

Fully quantitative aflatoxin determinations, which require the use of a standard of known concentration, can be carried out by a variety of techniques including: TLC, high performance liquid chromatography (HPLC), high performance thinlayer chromatography (HPTLC), and ELISA.

## HPLC

HPLC separates the chemical components of an extract on a very small-bore column filled with extremely finely ground packing material, eg 5  $\mu\text{m}$ , which has particles of uniform shape and size. This type of column presents very high resistance to the mobile phase and therefore high pressure pumps capable of pumping very smoothly at a pressure of 3,000 psi are required. A typical HPLC system for aflatoxin analysis would comprise: Two high pressure peristaltic pumps, system controller, solvent mixer, manual injection valve, autosampler, HPLC column, post-column derivitisation equipment such as a KOBRA cell, fluorescent detector, and a computer to control data acquisition and integrate and interpret the data. The equipment is costly, about £20,000, and is prone to leakages and other break-downs. Each sample takes between 20 and 30 minutes to analyse, and the system only becomes viable if an autosampler is used to run samples over-night. The main advantages that HPLC has is that it is capable of detecting very low levels of aflatoxin with good precision and accuracy. This makes it ideal for analysing foods where low aflatoxin limits are set.

## HPTLC

HPTLC is TLC using special high performance plates which are read by a computer-controlled scanning fluorodensitometer. Integration and other data handling are handled by computer software. The high performance plates not only result in better resolution of components, but they also allow tracks to be spotted at 5 mm intervals. A single 20x10 cm plate can analyse 12 samples in duplicate and calibrate on 5 standards. The capital cost of this equipment is similar to the HPLC, but it is usually more reliable. The main advantage of HPTLC is the high throughput of samples which is possible, it only takes about 4 minutes to scan a complete plate. The main disadvantage of HPTLC is that it is not capable of analysing

very low levels of aflatoxin with the required precision and accuracy. However, precision and accuracy matches that of HPLC at levels of aflatoxin above about 10  $\mu\text{g}/\text{kg}$ .

### **ELISA**

Kits are available commercially and these comprise a 96 well microtitre plate in which specific aflatoxin antibodies compete for aflatoxin which is either bound to the well, or is in the sample extract. The more aflatoxin the sample contains, the less anti-body binds to the well. The bound anti-body is detected by attaching an enzyme which is then visualised. The optical density of the wells is then read in a plate reader: the lower the reading, the higher is the level of aflatoxin.

The procedure is quite tedious and the test takes a total of about 4.5 hours, including 3 hours of incubations. However, up to 37 samples can be analysed in duplicate simultaneously, although it is more usual to only use a third of the plate and analyse 8 samples in duplicate. The plate reader is relatively inexpensive £2,000 manual, and £4,500 automatic, but the kits cost about £300 each (in the range £12.50 to £8.00 per sample), making running costs quite high. A major disadvantage of ELISA kits is that they must be stored in a refrigerator, and they can easily be destroyed in transit from the supplier.

### **Criteria for Selecting Methods**

An ideal assay procedure for aflatoxin would be easy to perform (simple), robust, rapid, precise, accurate, have a low detection limit, have low capital and running costs, and be fully automated. Needless to say, no assay procedure currently available can satisfy all these criteria.

Consequently, a compromise must be made which best suits the resources and objectives of the laboratory.

Some of these criteria are assessed for: HPLC, HPTLC, TLC, and ELISA in Table 2.

**Table 2. Comparison of Quantitative Aflatoxin Techniques**

Criteria	TLC	HPLC	HPTLC	ELISA
Ease of use	Good	Good	Good	Poor
Speed, total	Moderate	Poor	Moderate	Poor
Speed/sample	Moderate	Poor	Very Good	Good
Cost, capital	Low**	Very High	Very High	Low
Cost, running	Low	Low	Low	High
Accuracy	Moderate	Very Good	Very Good*	Good
Precision	Moderate	Very Good	Very Good*	Good
Sensitivity	Moderate	Very Good	Good	Good
Robustness	Good	Poor	Good	Poor

\* = Except at low levels below 10 µg/kg

\*\* = Excluding laboratory facilities such as a fume cupboard

An assessment of semi-quantitative techniques available at the UNIDO laboratory is given in Table 3.

**Table 3. Comparison of Semi-Quantitative Aflatoxin Techniques**

Criteria	TLC Screen	EZ-Card Test	Easi-Assay
Ease of use	Good	Excellent	Very Good
Speed, total	Moderate	Excellent	Moderate
Speed/sample	Excellent	Excellent	Poor*
Cost, capital	Low**	Low (None)	Low
Cost, running	Very Low	Moderate	High
Accuracy	Good	Good	Good
Precision	Moderate	Very Good	Very Good*
Sensitivity	>=1 ppb	<5> ppb	1 ppb
Robustness	Good	Poor (0-4°C)	Poor (0-4°C)

\* = Could be improved with vacuum manifold

\*\* = Excluding laboratory facilities such as a fume cupboard

Assessment of the various criteria is often subjective, for example one analyst might find a method easy to perform, whilst another might experience difficulties.

Another important factor in the choice of a method is whether there is any technical support for the capital equipment locally or in-country. This would be especially true for the less robust equipment such as HPLC. The supply of consumables and especially kits which need to be kept refrigerated, would also need to be checked.

#### **Confirmation**

It is essential that confirmatory tests be carried out if a mycotoxin is thought to have been detected, especially when a new commodity, or new source of commodity is being analysed. Failure to do this could easily lead to false-positive results, i.e. an interfering compound is mistaken for a known mycotoxin.

Prepared by M J Nagler

18 June 1995

**Annex 4E).****SAMPLE COLLECTION AND SAMPLE PREPARATION, NATURE OF AFLATOXIN  
DISTRIBUTION AND PRACTICAL SOLUTIONS****Importance of the Sample Collection Step**

The variance associated with an aflatoxin result is a combination of the variances associated with each stage of the determination: namely, sample collection, sub-sampling (sample division) and analysis.

$$V_{\text{total}} = V_{\text{sampling}} + V_{\text{sub-sampling}} + V_{\text{analysis}}$$

Sample collection is usually the major source of variance and sampling is therefore a critical stage in the process of determining levels of aflatoxin in a commodity. The primary sample needs to be 'representative' of the whole batch, so that the level of aflatoxin that is found in the sample reflects that present in the batch.

**Aflatoxin Distribution**

The problem is that aflatoxin is not usually distributed evenly throughout a batch: only a small percentage of particles are contaminated, but these can be contaminated a very high level. For example, when 240 x 100 g incremental samples of maize were analysed separately, instead of combining them together to give a primary sample, it was found that 55% of the increments did not contain aflatoxin and about 80% of the increments contained less than the mean of 40 ppb. This highly 'skewed' distribution is shown in Chart 1. When computer simulations were performed on the data, it was found that at least 100 increments, giving a primary sample of 10 kg, were needed to achieve a result which was approximately normally distributed about the mean. It is known that other commodities, such as copra and groundnuts, have similar highly skewed distributions of aflatoxin, and require the collection of a large number of incremental samples to give a large primary sample. Studies by Steiner strongly indicate that the distribution of aflatoxin in pistachios is also skewed: he estimated that only one nut in about 5,000 was contaminated. However, levels of aflatoxin in single nuts could be extremely high.

One naturally contaminated pistachio nut contained aflatoxin at 1,400 mg/kg (ppm).

As a guide, it has been recommended that a representative sample should contain at least 40,000 particles, so the bigger the particle size the heavier will be the sample required. Using this concept, commodities can be divided into three types, with Type 3 being divided into three classes as described in Table 4. below.

**Table 4. Minimum Sample Size to Obtain a Representative Sample (Assuming 40,000 Particles)**

Type	Definition/Commodity	Minimum Sample Weight
1	Very small particles or droplets eg milk, milk products, and vegetable oil	500 g
2	Particles of intermediate size, eg finely ground meals and flours, peanut butter	3 kg
3a	Small grains, eg wheat, barley, oats, rice and sorghum	5 kg
3b	Intermediate grains, eg maize, cottonseed	10 kg
3c	Large grains, eg groundnuts, tree-nuts including pistachios	20 kg

#### **Sampling Plans, including Pistachios**

A sampling plan stipulates the sample size required for a given commodity, and usually stipulates how it should be collected, eg the minimum number of increments from throughout the batch. Some sampling plans, such as the USA sampling plan for groundnuts, is very complex. Not only does it require the collection of 3 x 21.8 kg from each 20 tonne batch, but it also stipulates how to decide whether to accept or reject the batch on the basis of the aflatoxin results.

Sampling plans for pistachios appear to be less rigorous in terms of sample size, but the limits can be very low. In the UK the limit is only 4 µg/kg (ppb) total aflatoxin for retail packs, and the importers must reject all batches exceeding a aflatoxin level of 10 µg/kg. Some of the sampling plans are listed in Table 5.

**Table 5. Sampling Plans for Pistachios**

Country	Pistachio	Number of increments	Minimum sample weight	Batch size tonnes	Aflatoxin limit $\mu\text{g}/\text{kg}$
UK	kernel	20	1.5	<25	4 (10)
UK	in-shell	20	3.0	<25	4 (10)
USA	in-shell	?	21.8	34	25

The UK Regulation appears to be quite generous in terms of sample weight, but the aflatoxin limit is set very low.

### Sample Preparation

Once a representative sample has been collected, it is important to keep the integrity of the sample as sample size is reduced to that of the analytical sample. To do this the primary sample must be ground and mixed or homogenised prior to sample division.

For pistachios, a vertical cutter mixer fitted with a cutter blade is used in the UNIDO/GTS aflatoxin laboratory to prepare an homogeneous paste on the 10 kg scale (SOP 3A). Pistachios in-shell are also prepared in the same mill, but this time it is fitted with a mixing insert (SOP 3B). Following milling it is possible to separate the kernels from shell by sieving through a 2 mm screen. The separated kernels are then formed into a paste as in SOP 3A.

These procedures are similar to those stipulated in the UK Regulations, except that the regulations stipulate tumble mixing in-shell pistachios for 60 minutes after the sieving step. The rationale for this is not known.

### Recommendations

1. The sample weight to be collected for monitoring batches of pistachio kernels or whole nuts in-shell should be 10 kg.
2. The primary sample should be composed of at least 20 incremental samples each weighing 500 g drawn from throughout the batch.
3. Sample preparation according to SOPs 3A and 3B should be followed

*Prepared by Martin Nagler, 18 June 1995*



Annex 5: List of People Met

No.	Name	Organisation/Address
1	Prof. Dr. Fatih Yildiz	National Project Director Department of Food Engineering Middle East Technical University İnönü Bulvarı 06531 Ankara, Turkey Tel: 312 2101000 Fax: 312 2101270
2	Mr Hasan Ozturkmen	President
3	Mr Ekrem Özaslan	Chairman
4	Mr Ahmet Adali	Deputy General Manager
5	Mr Huseyen Senturk	Director of Processing
6	Mr Mustafa Özekin	Deputy Director of Processing
7	Mr Fatih Turnalar	Food Science & Technology Engineer
8	Mrs Aysel Kasakolu	(Manager of Aflatoxin Laboratory)
9	Mrs Meryem Bolatdenirci	Agricultural Engineer
10	Mr Mehmet A Elma	(Trained in aflatoxin analysis)
11	Mr Ali Özerk	Laboratory Analyst
12	Mr Nihat Dindaroglu	Agricultural Engineer Laboratory Attendant Electrician Güneydogu Union of Agricultural Sales Cooperatives, P.O. Box 70 Gaziantep Turkey Tel: 342 2324290 Fax: 342 2308692

12	Prof. Dr. Mehmet D.	Head, Food Engineering
13	Öner	Department
14	Dr Ali Riza Tekin	Lecturer
15	Dr Fahrettin Gögüs	Lecturer
16	Ms Birgül Akar	Post-graduate
	Ms Zehra Estekin	Under-graduate/Trainee Analyst at the UNIDO Laboratory. University of Gaziantep Gaziantep, Turkey Tel: 342 3601105
17	Mr Andrew Picken	First Secretary Science and Technology
18	Ms Jale Anagnan	Visitors to Britain Scheme British Council in Turkey Kirlangic Sokak No:9 Gaziosmanpasa 06700 Ankara, Turkey. Tel. (312) 468 6192-99 Fax. (312) 427 6182
19	Dr Hüseyin Tekin	Director, Pistachio Research Inst. P.K. 32 27001 Gaziantep Tel. (342) 338 3052 Fax. (342) 338 1464

**Annex 6: Summary of Visits****Meeting 1: British Council, Ankara**

Date: 25 April 1995, 11.00 hrs.

Accompanied by Prof. Dr. Fatih Yildiz (National Project Director).

Met: Mr Andrew Picken (First Secretary Science & Technology)  
Ms Jale Anagnan (Link Visitors to Britain Scheme)

Purpose of visit: To explore the possibility of British Council assistance in future technical training and/or institutional links between UK and Turkish project collaborators.

Outcome: Ms Anagnan described the Links programme which can provide for short visits between Turkey and the UK. Turkish visitors to the UK can apply for a grant to provide a contribution to modest accommodation, meals, and transport to and from UK airports. It does not cover airfares nor any fees or other expenses in the UK. The specialists to Turkey scheme is for invited visitors from equivalent British institutions. The visitor is provided the cheapest concessicnary airfare to Turkey and the host institution must meet the visitor on arrival and pay for all accommodation and food.

Mr Picken explained that Turkey is no longer classified as a 'developing country' by the UK's Overseas Development Administration and hence does not receive bi-lateral aid. Therefore there are no longer any project-related training scholarships. Demand for British Council Scholarships was very high and only the very ablest applicants were successful in gaining a scholarship.

Mr Picken suggested that the most likely source of funds for any future Project support (apart from UNIDO) was the European Union. In six to nine months time the Fourth Protocol Aid to Turkey would come on-line and there was also a new Mediterranean Programme being administered from Brussels. When, as seems likely, Turkey becomes a signatory to the European Customs Union then trade would expand rapidly. Quality issues, such as aflatoxin contamination of pistachio nuts, would then increase in importance, and European funds may become more likely to be channelled towards quality control projects. The Med-Campus programme, which Turkey already makes good use of, could also provide institutional links.

We did visit the European Union building in Ankara after this meeting, but we were unable to arrange an appointment with anyone from the Aid Section at short notice.

Mr Picken also mentioned that the British Embassy had a particular interest in South Eastern Turkey, it being the poorest, and that the First Secretary, Commercial Section, Mr Stephenson, was currently visiting Gaziantep.

Some potentially useful leads were picked up and the National Project Director will follow these up.

#### **Meeting 2: National Project Director**

Prof. Fatih Yildiz kindly picked me up from the airport in Ankara and took me to my hotel. We discussed means whereby the UNIDO aflatoxin laboratory could develop and become recognised internationally agreed that it was important that the laboratory should establish links with reference laboratories in Europe, particularly the UK and Germany. I suggested that the Natural Resources Institute would be a suitable UK laboratory.

We discussed the proposed aflatoxin training course that I was scheduled to give towards the end of my visit. Prof. Yildiz said that the course should be designed for laboratory technicians who were already carrying out aflatoxin analyses, or who planned to do so in the near future. Few, if any, of the trainees would be fluent in English, so it would be important to prepare the course material in good time and get it translated into Turkish.

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## BACKSTOPPING OFFICER'S COMMENTS

1. The report summarizes the activities carried out by the consultant during the second phase of his assignment where he has been responsible for commissioning a pistachio nut aflatoxin quality control laboratory at the Guneydogu Union of Agricultural Sales and Cooperatives (GTS). The activities consisted essentially in the installation of the equipment, test of the methodology previously proposed by the Natural Resources Institute (NRI) and in training the laboratory staff plus representatives of other laboratories and pistachio producing companies.

2. In addition to some operational problems faced as a consequence of the import of the laboratory equipment as for instance the non compliance of specifications by the supplier of some equipment components (partially solved before the mission)<sup>1</sup>, the consultant have described in detail all essential Laboratory Standard Operating Procedures (SOPs) which are presented in the report as Annex 3. *The SOPs would have to be really followed by the laboratory personnel and management.* Also some important general laboratory information on toxin analysis have been presented under Annex 4, particularly from Annex 4C to Annex 4E. *It is also recommended that they be carefully taken into account during laboratory operation.*

3. The report also presents important conclusions and recommendations, respectively under chapters IV and V. Among them, it is worthwhile to point out that: (i) The staffing of the laboratory will have to be completed in line with the short job descriptions presented under chapter I; (ii) some complementary equipment will have to be purchased as described briefly under items V c), d), i) and j). *Before deciding on the purchase of the equipment for quantitative aflatoxin analysis it is essential that Annex 4D, particularly Table 2., be examined in detail. There, a very interesting and useful comparison among different quantitative aflatoxin techniques is provided;* (iii) The laboratory staff will have to become independent of GTS, and a policy statement agreed at Board level, if the aim is to meet European EN45001. *In order to have international projection which would be directly linked to the Government policy of increasing foreign exchange earning through increasing pistachio production and processing, it seems essential that this last recommendation be also followed by the national authorities.*

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<sup>1</sup> In the case of ELISA (Enzyme-Linked Immuno-Sorbent Assay) kits neither the supplier nor the recipient counterpart have accepted responsibility for not having been kept at the proper temperature during transportation and while stored.