This manual was prepared by the Ministry of Agriculture, Livestock and Supply (MAPA) of Brazil, through its Reference Laboratory for Mycotoxins (Laboratory for Quality Control and Food Safety – LACQSA/LAV-MG) as a training material for the Food and Agriculture Organisation of the United Nations (FAO) Training Course “Mycotoxin Analysis and Laboratory Management” to be held at Kenyan Research Centre during the period 12th to 23th April 2004, as part of the Global Project “Enhancement of Coffee Quality regarding Mould Formation”.

Mycotoxin analysis and laboratory management

“MYCOTOXIN ANALYSIS AND LABORATORY MANAGEMENT”

Manual for the training course organised jointly by the Food Agriculture Organisation (FAO), the Ministry of Agriculture Livestock and Supply (MAPA) of Brazil, at the Kenyan Coffee Research Foundation (CRF), Nairobi, Kenya, April 12th - 23rd.

Ministério da Agricultura, Pecuária e Abastecimento

LACQSA

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The LACQSA/LAV-MG staff and to everyone who indirectly contributed for the fulfilment of this work

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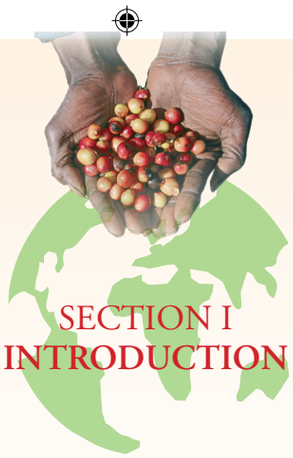

SECTION I
INTRODUCTION

Regulations and guidelines on ochratoxin A in food have been laid down in many countries with levels ranging from 2 ng/g to 50 ng/g. It is envisaged that the European Community Commission – considering the fact that the presence of ochratoxin A in foodstuffs represents risk to human health will in the future lay down regulations for the presence of ochratoxin A in green and roasted coffee, along with sampling plans and method performance criteria, as a modification of EC directives no. 2002/472/CE and 2002/26/CE, respectively.

This scenario determines, for the coffee producing countries, the necessity of having in place validated analytical methods, sampling plans and quality assurance system (QAS) to assess whether the coffee exported meets the standards of the importing countries.

This manual was prepared by the Ministry of Agriculture, Livestock and Supply (MAPA) of Brazil, through its Reference Laboratory for Mycotoxins (Laboratory for Quality Control and Food Safety – LACQSA/LAV-MG) as a training material for the Food and Agriculture Organisation of the United Nations (FAO) Training Course "Mycotoxin Analysis and Laboratory Management" to be held at Kenyan Research Centre during the period 12th to 23th April 2004, as part of the Global Project "Enhancement of Coffee Quality regarding Mould Formation".

This instruction literature was worked out by E. A. Vargas, L. Castro, E. A. Santos, C. M. G. Silva, S. S. Amorim, R. A. Preis and T. A. Sá, based on the ISO standard 17025 itself and LACQSA/LAV-MG experience on implementation of a QAS also based on ISO standard 17025 and on method validation directives.

The purpose of this manual is to provide guidance and examples to those laboratories wishing to carry out mycotoxin analysis under a QAS regime. It must be emphasised that every laboratory quality assurance system shall be designed and implemented taking into account the laboratory scope and the "fit for purpose" ideal of a QAS.

SECTION II
IMPLEMENTATION OF THE QUALITY SYSTEM

The Laboratory must have a minimum and comparable quality assurance system (QAS) in place, use validated methods and demonstrate its analytical competence by taking part in proficiency testing to ensure that data of demonstrable quality are being generated. There are different levels of sophistication of quality control (QC) systems and, for a laboratory in a developing country, initially, a very simple QC system can be used to form a basis for a future third party audited system, fully in compliance with ISO 17025 (Gilbert 2002). According to the "Guide to Quality in Analytical Chemistry" (EURACHEM/CITAC 2002), *the QAS should describe the overall measures that a laboratory is expected to take to ensure the quality of its operations, which might include:*

- *A quality system;*
- *Proper laboratory environment;*
- *Well instructed, trained and skilled staff;*
- *Training procedures and records;*
- *Equipment properly maintained and calibrated;*
- *Quality control procedures;*
- *Documented and validated methods;*
- *Traceability and assessment of measurement uncertainty;*
- *Check and report procedures;*
- *Preventative and corrective actions;*
- *Proficiency testing;*
- *Internal audit and review procedures;*
- *Procedures regarding complaints;*
- *Requests for reagents, calibrants, measurement standards and reference materials.*

Implementation of the QAS, based on ISO 17025 (1999), provides reliability on the data generated by the Laboratory and the possibility of their mutual international recognition by the importing countries (Garfield *et al* 2000).

This section was written based on ISO 17025 (1999) – "General Requirements for the Competence of Calibration and Testing Laboratories" and on the already implemented Quality Assurance System of the Laboratory for Quality Control and Food Safety (LACQSA/LAV-MG) - the

reference laboratory for mycotoxin analysis of the Ministry of Agriculture, Livestock and Supply of Brazil (MAPA 2001a).

II.1. COMPLIANCE WITH MANAGEMENT REQUIREMENTS

II.1.1. ORGANISATION AND MANAGEMENT

The laboratory shall be organised and operated so as to meet the requirements of the International Standard ISO 17025 (1999).

The laboratory shall:

- Have managerial and technical personnel;
- Have arrangements to ensure that its management and personnel are free from pressures that may adversely affect the quality of their work;
- Have policies and procedures to ensure the protection of its customers' confidential information and proprietary rights;
- Have polices and procedures to prevent involvement in any activities that might diminish confidence in its competence, impartiality, judgement or operational integrity (*Note: customers' confidential information and proprietary rights can be ensure by the following procedures: access control to the installations and analytical results, confidentiality during sample preparation, sign of confidentiality and compromise terms by the laboratory staff, and procedure of keeping and transmission of results);*
- Define the organisation and management structure (*Figures 1* and *2*);
- Specify the responsibility, authority and interrelationship of all personnel;
- Provide adequate supervision of testing staff;
- Have a technical management, responsible for all technical and administrative operation and for provision of the resources needed to assure a good management performance and a quality management with defined responsibility and authority to assure the quality system to be permanently implemented and followed, with access to the immediate supervision, where the decisions on the Laboratory resources are taken;
- Appoint deputies for key managerial personnel.

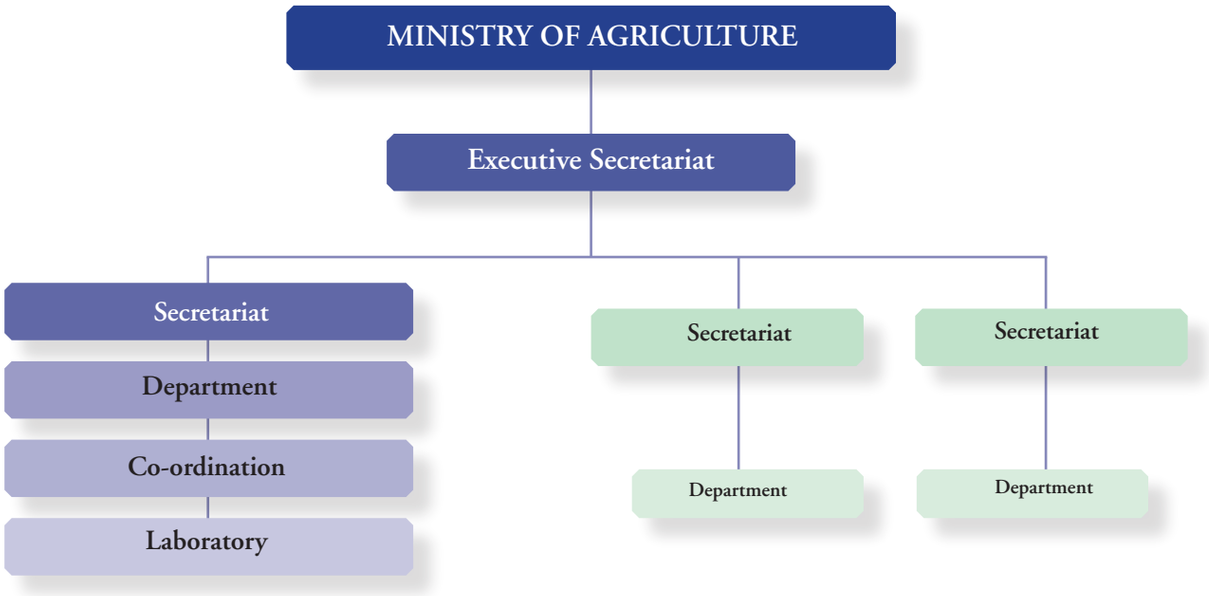

Figure 1: Example of LACQSA organisation chart

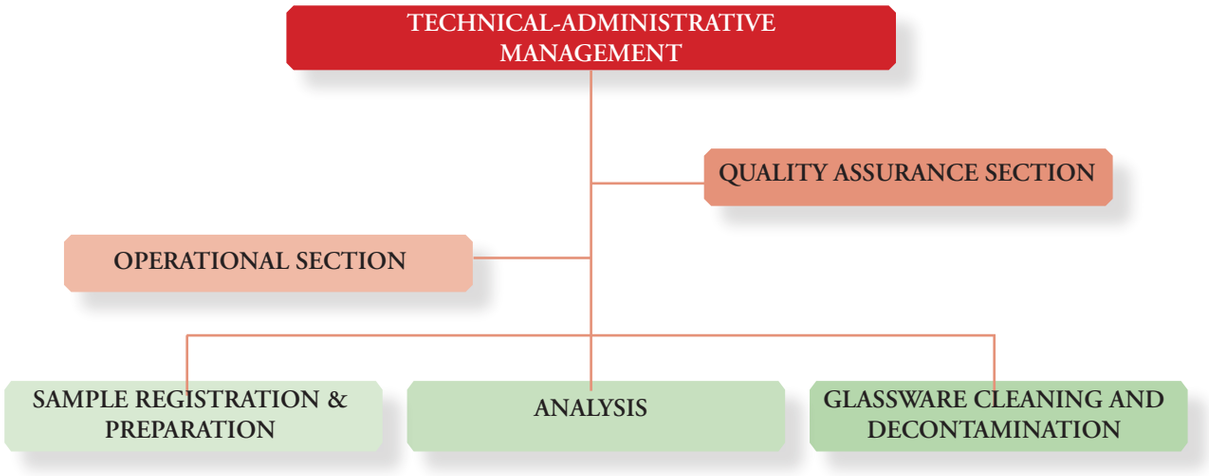

Figure 2: Example of organisation of LACQSA internal structure

II.1.2. QUALITY SYSTEM

The laboratory management shall establish, implement and maintain a quality system appropriate to the scope of its activities. The laboratory shall document its policies, systems, programmes, procedures and instructions to the extent necessary to enable the laboratory to assure the quality of its analytical results. The documentation utilised in this quality system shall be communicated to, understood by, implemented by and available to appropriate personnel.

The quality policy should be defined aiming at ensuring a high quality standard of the services rendered by the laboratory, committing the Laboratory with good professional practices, with analytical quality and reliability of the results as well as satisfaction of the customers. The organisation shall confirm the compromise to assure resources and personnel to follow the laboratory policies and procedures, in compliance with the standard ISO 17025 (1999). The Laboratory staff have to be aware of the contents of the Quality Manual, and follow its policy, procedures and related quality documents, unconditionally.

II.1.2.1. DOCUMENTATION STRUCTURE

The documentation shall comprise a Quality Manual (QM), Standard Operational Procedures (SOP), instructions, registration forms, lists, reports and plans. The documentation structure shall be defined so as to facilitate traceability of the analytical result and all information related to the quality system. The Quality Manual refers to the SOP's, which in turn refer to the instructions, registration forms and relevant reports, and the registration forms refer to the SOP's utilised for generation of the data (MAPA 2001a).

A hierarchical structure of the documentation should be established (*Figure 3*) as well as the organisation of the principal quality documents, as illustrated in *Figures 4* and *5* (MAPA 2001a).

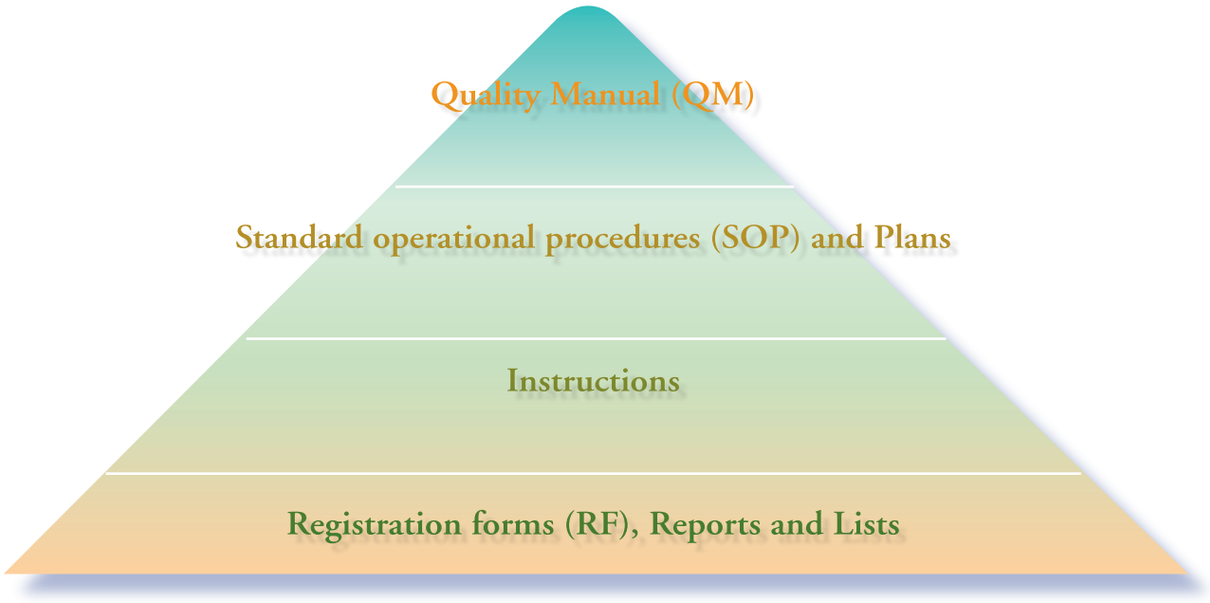

Figure 3: Example of hierarchical structure of the LACQSA quality system documentation

II.1.2.2. QUALITY MANUAL

The Quality Manual is the master document, and only one is required independently of the size of the organization, and it must be maintained updated. The Quality Manual shall establish the policies related to management and technical aspects of the Laboratory, in accordance with the Standard ISO 17025 (1999) and shall contain minimum contents, such as (MAPA 2001a):

- Laboratory scope;
- Reference to major pieces of equipment utilised;
- Reference to calibration and/or analytical procedures utilised;
- Charts defining the organisation of the laboratory;
- Relation between management, technical operations, support services and quality control;
- A list of laboratory authorised signatories;
- A job description of key managerial and technical staff.

Figure 4: Example of principal documents established at LACQSA for compliance with management requirements – ISO 17025

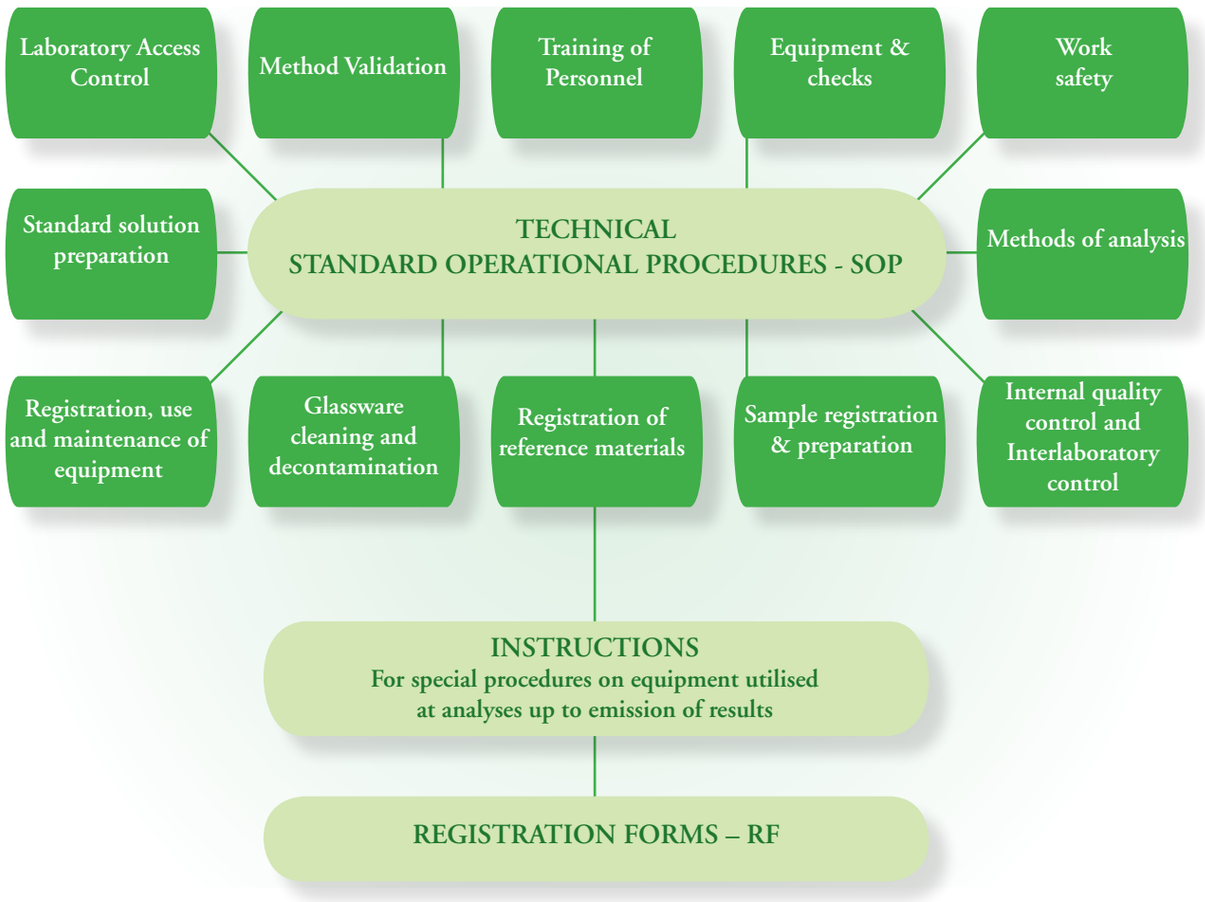

Figure 5: Example of principal documents established at LACQSA for compliance with technical requirements – ISO 17025

I.1.2.3. PROCEDURES, INSTRUCTIONS AND RECORDS

The SOP's describe the activities related to management and technical requirements, according to ISO 17025 (1999), to the extent necessary to enable the laboratory to assure the quality of the analytical results.

Each individual analytical method shall be described in the form of SOP. The minimum contents necessary in an analytical method are:

- Applicability (which mycotoxins it covers and which matrices);
- Safety aspects;
- Principle of the method;
- Reagents and material;
- Method (extraction, clean-up and quantification steps);
- Method performance characteristics;
- Quality control procedures;
- Report of results.

The Instructions provide, in a schematic way, detailed procedures for use of the equipment and development of other activities, as exemplified in *Figure 6*.

MINISTRY OF AGRICULTURE, LIVESTOCK AND SUPPLY OF BRAZIL *LABORATORY FOR QUALITY CONTROL AND FOOD SAFETY - LACQSA/LAV-MG*	*Code: IU – XX* *Edition: XX* *Review: XX* ***Date: XX***
Instruction of use ***Title:*** *Electronic Scale*	*Page: X of Y* *Edited by: XX* ***Approved by: XX***

Specifications: Brand: CHYO, Model: MJ-3000, Laboratory File No.: 004

Operational cautions:

1. Make sure the scale is installed on a flat level surface, free of vibration and air current (keep air conditioner off).
2. The scale should not be located near a heating equipment or exposed to sun rays or warm irradiation (furnace, oven, flames, heaters).
3. Be sure no liquid penetrate the weighing plate or the scale.
4. Don't weigh samples or recipients with electrostatic charges, since they will produce disturbances to the scale and consequently to the weighing.
5. Internal calibration of the scale should not be tampered with.
6. The Manual Instructions should be read whenever necessary.

Cautions for cleaning of the scale
1. Before start cleaning the scale, be sure it is unplugged from the power outlet.
2. Never use organic solvents, aggressive detergents, solvents or similar.
3. Don't let any liquid enter the scale.
4. Carefully remove any remaining samples/reagents with an appropriate brush.
5. Clean the scale with flannel or a soft brush.

Procedure for use
6. Connect the A/C power plug into the scale and into the proper power source;
7. Please wait until the display shows from 9999999 until 0000000 and "s" at the lower left area;
8. Press the key "ON/Stand-by" and wait for 30 minutes to allow scale adaptation to the environmental conditions and consequent stabilisation;
9. Press the scale key "TARE";
10. Make the scale performance check according to SOP 023 "Checking of performance of scales", and record the date, time, name of analyst, weighing values and approval as per RF 015 "Performance of pieces of equipment – Control of scales", which can be read close to the scale;
*11. Place the recipient in which the sample is to be weighed in the centre of the weighing plate and wait until the display shows steadily the symbols "< + *", indicating that the scale is stabilised;*
12. Press the scale key TARE;
13. Place the sample/reagent in the recipient and wait until the scale is stabilised;
14. Read the weight of the substance when " *"appears on the display;*
15. Write down the weight shown and remove the sample/reagent;
16. Press the key "ON/Stand-by" after completion of the weighing;
17. Disconnect the A/C power plug from the power outlet;
18. Properly clean and cover the scale.

Approval signatures:

Figure 6: Examples of LACQSA instruction of use.

I.1.2.4. PLANS, LISTS, AND REPORTS

The content of the plans, lists and reports must be suitable for the type of activities developed. The plans contain minimally a schedule of the activities to be performed (for study plans see section II item 2.3.2 and for audit plan see section II item 1.9). The list contains a description of the staff members, equipment or documents with their respective identification codes. The reports must be written down/elaborated or filled out after completion of the activities and must contain the introduction, material and methods, period, technicians involved, results and conclusions. Examples of these documents are shown in *Figure 7* (MAPA 2001a).

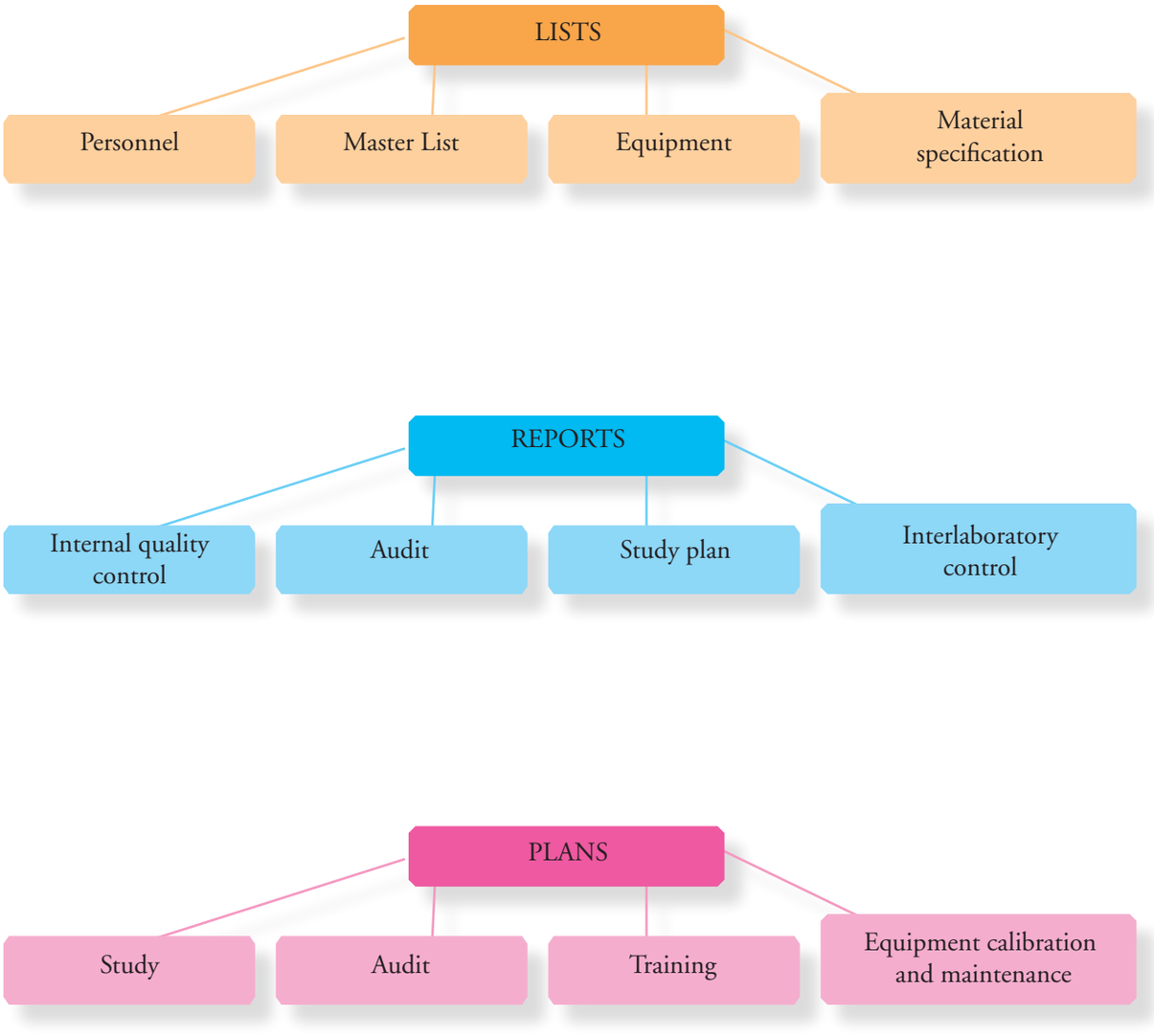

Figure 7: Examples of lists, reports and plans related to the LACQSA Quality Assurance System

II.1.3. CONTROL OF DOCUMENTS AND REGISTERS

According to ISO 17025 (1999) the documents should be controlled and procedures should be established for approval, emission and alterations in documents. The quality system documents, both management and technical, should be listed in a document control such as the Master List (*Figure 8*) and identified in a unique form, with date of issue, date of edition, review, pages, total number of pages, emitter authority and distribution of controlled copies (MAPA 2001a).

The technical and management records must be legible, in ink, made at the moment when the procedures are performed and should be stored and preserved so as to assure their brief recovery, in appropriate condition to ensure their integrity against damage, deterioration or loss; they should be kept safe and confidential and shall be identified, collected, indexed, accessed, filed, stored, maintained and settled so as to assure traceability of the activities performed at the laboratory, enabling the analysis or procedure to be repeated under conditions as close as possible to the original conditions (ISO 17025 1999, MAPA 2001a).

According to ISO 17025 (1999) the procedures adopted shall ensure that:

- Authorised and updated documents are available at the locations where the activities are performed;
- Documents are periodically analysed and reviewed whenever necessary, assuring adequacy and conformance to the ISO standard requirements;
- Obsolete documents are promptly removed from the locations where they are utilised, identified with a double diagonal line and filed (original copy) or disposed (other copies).

The laboratory shall retain original observations, derived data and sufficient information to establish an audit trail, calibration records, staff records and a copy of each test report issued, for a defined period.

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MASTER LIST	*Emitted by:* *Approved by: Laboratory manager and Quality manager*

Code	*Title*	*Ed*	*Rev*	*Ed*	*Rev*	*Date/Signat.*	*Ed*	*Rev*	*Date/Signat.*	*Ed*	*Rev*	*Date/Signat.*
RF – 001	*Samples/analysis Record*	*10*	*03*									
RF – 002	*Equipment record*	*11*	*03*									
RF – 003	*Analysis follow up*	*08*	*05*									
RF – 004	*Preparation of standard solutions*	*05*	*03*									
RF – 005	*Training of personnel*	*02*	*01*									

Code	*Title*	*Ed*	*Rev*	*Ed*	*Rev*	*Date/Signat.*	*Ed*	*Rev*	*Date/Signat.*	*Controlled Copy*
IU – 001	*Scale*	*02*	*01*							*Laboratory*
IU – 002	*Mill*	*02*	*01*							*Laboratory*
IU – 003	*Separation funnel agitator*	*02*	*01*							*Laboratory*
SOP – 001	*Elaboration and control of documents*	*05*	*04*							*Quality file*
SOP – 002	*Ochratoxin A Analysis*	*02*	*01*							*Laboratory*
SOP – 003	*Interlaboratory control*	*03*	*02*							*Quality file*
SOP – 004	*Intralaboratory control*	*02*	*01*							*Quality file*
SOP – 006	*Sample Preparation*	*03*	*02*							*Sample preparation*
SOP – 007	*Training of personnel*	*04*	*03*							*Quality file*

Figure 8: Example of LACQSA master list

II.1.4 REVIEW OF REQUEST, TENDER AND CONTRACT

According ISO 17025 (1999) the laboratory shall establish and maintain procedures for review of requests, tenders or contracts, which shall ensure that *(Figure 9)*:

- The requirements including the methods to be utilised are adequately defined, documented and understood;
- The laboratory may have the capability and resources to meet the requirements;
- The appropriate test method is selected and capable of meeting the customers' requirements.

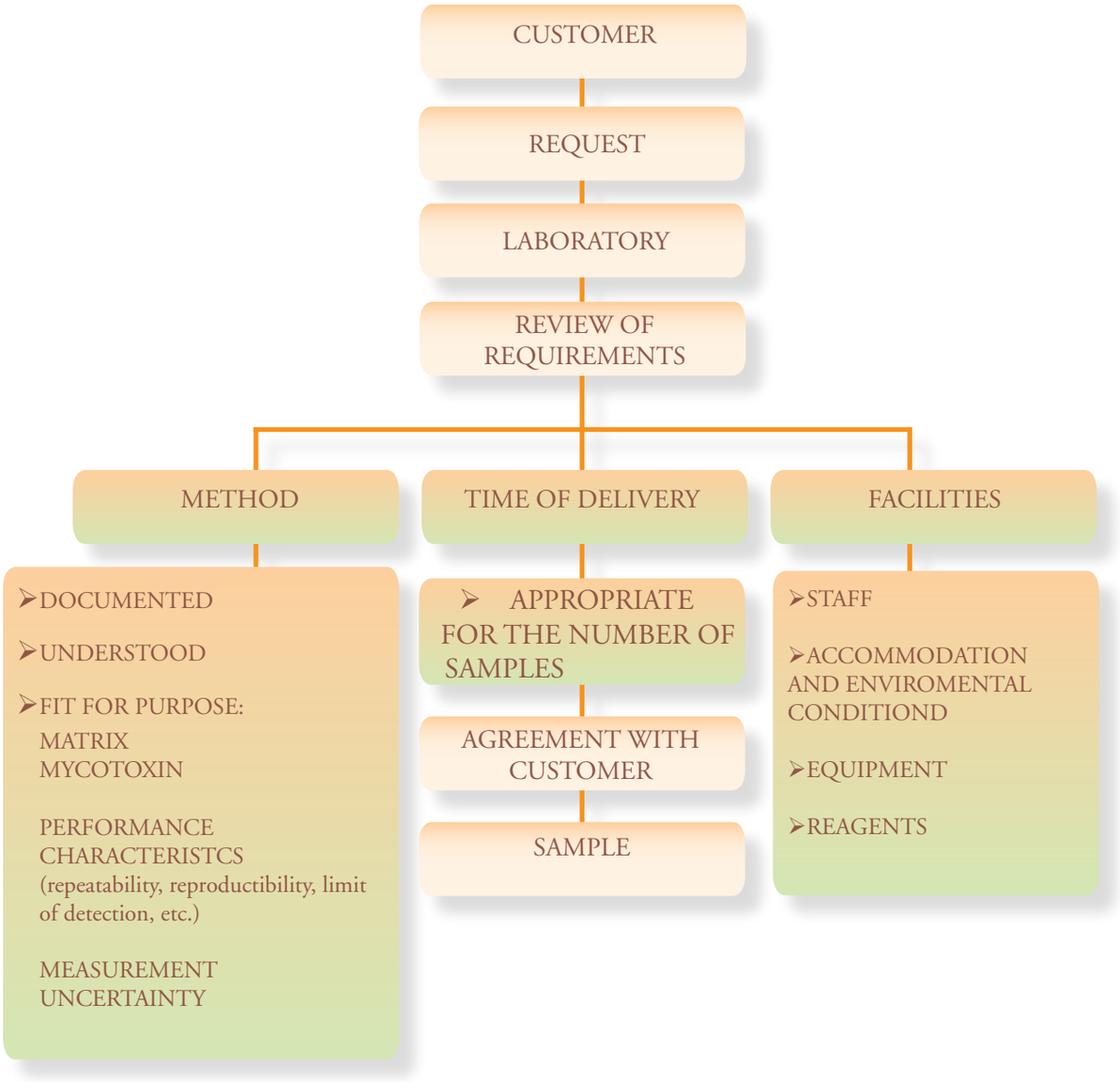

Figure 9: Review of request flow chart

The Laboratory shall keep the documentation related to the request, the records of the review and the final agreement with the customer (MAPA 2001a). The procedures to be adopted at sample reception are described in the section II.2.7. Handling of Samples.

II.1.5. SUBCONTRACTING OF TESTS

According to ISO 17025 (1999), when a laboratory has to subcontract work (when the use of facilities or equipment from another Laboratory is needed to complete the analytical request), this shall be entrusted to a competent subcontractor.

The Quality Manual shall include the mechanism for assessing and selecting subcontractors. The results from a subcontractor should never be presented as results from the contracted Laboratory.

II.1.6. PURCHASE OF SERVICES AND SUPPLIES

The laboratory shall have policies and procedures to ensure that purchased equipment, material and services (that affect the quality of the tests) comply with specified technical requirements. In the case of measurement equipment (scales, thermometers, micropipettes, volumetric glassware etc.), these shall have a certificate of calibration given by a laboratory capable to prove the traceability to the SI (Système International). The purchase order must include, when of interest to the Laboratory, a guaranty, installation description, training and material specifications for prompt operation and use or a user's manual. The laboratory shall ensure that purchased and consumable materials that affect the quality are not utilised until they have been checked as complying with the standard specifications or requirements defined in the methods for the test concerned and on the specification list. In case of a piece of equipment, it shall be received, checked and registered, also with a specific Laboratory code number. The materials must be stored in the premises of the Laboratory, grouped according to their nature: either solvents, solid reagents, or other materials such as TLC plates and chromatographic columns. The reagents shall be organised taking into account their expiring date. For products that demand special storage conditions (reference materials, standards of mycotoxins and immunoaffinity columns) the manufacturer's recommendations should be followed. Services of third parties to be hired by the Laboratory should be previously selected. At selection, special consideration should be given to adequacy of specifications, term of delivery, price, guaranty of the service rendered and attendance to the customer. The laboratory shall evaluate the suppliers of critical services which may affect the quality of tests and maintain records of these evaluations and list the ones approved (ISO 17025 1999, MAPA 2001a).

II.1.7. SERVICE TO THE CUSTOMER AND COMPLAINTS

The laboratory shall provide to the customer all information requested and allow him to monitor the performance of the laboratory in relation to the work contracted.

The laboratory shall have a policy and procedure for resolution of complaints received from customers. Such complaints shall be recorded and investigated so that preventive or corrective actions may be taken, according to section II.1.8. Control of Nonconforming Work, Corrective and Preventive Actions.

II.1.8. CONTROL OF NONCONFORMING WORK, CORRECTIVE AND PREVENTIVE ACTIONS

The laboratory shall have a policy and procedures to be implemented whenever any aspect of its testing work or the results of this work do not comply with its own procedures or the agreed requirements of the customer (ISO 17025 1999).

The importance of the nonconforming work and its consequences on the analytical result emitted or to be emitted shall be evaluated and the nonconformances may be classified either as major, (if characterised as systematic failures, that directly influence the quality of the results obtained) or minor (when it does not directly influence the analytical result, characterised as isolated documental failures, whose procedure adopted is correct but is not duly documented). If the results obtained or the procedures have been affected to an extent to cause errors or deviations on the results emitted, corrective actions should be immediately implemented, the work should be interrupted and it shall only be resumed after evidence of elimination of the non-conformances. The customer should then be notified, if necessary. The responsibility for authorizing the resumption of the work shall be defined (MAPA 2001a).

The procedure for a corrective action shall start with investigation to determine the root cause of the problem, named cause analysis. The laboratory shall determine the potential corrective actions, select and implement those actions most likely to eliminate the problem and to prevent recurrence (*Figure 10*). The degree of the corrective action should be appropriate to the magnitude of the nonconformance. The terms for implementation and persons in charge should be defined. The dead line for implementation of corrective actions must be stipulated. The implementation of actions should be monitored so as to assure fulfilment of the proper actions and elimination of the nonconformances. In case the actions proposed have not been satisfactorily implemented or were not effective, a new action should be proposed. Preventive actions shall be taken if improvement opportunities are identified (ISO 17025 1999).

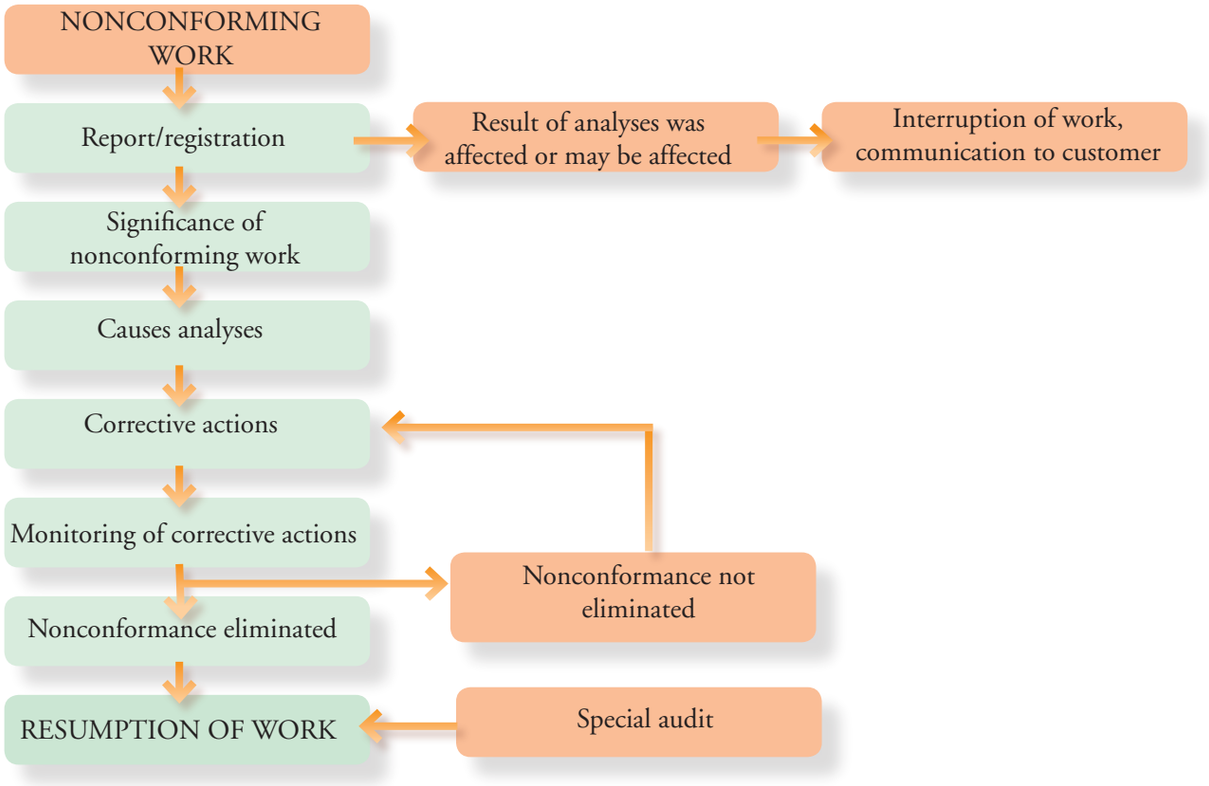

Figure 10: Example of LACQSA procedure established for control and correction of non-conforming works

II.1.9. INTERNAL AUDITS

Internal audits shall be periodically carried out, according to predetermined schedule, with the purpose of assuring the performance of the laboratory quality system, to improve it and verify its conformance with the standard ISO 17025, and with the Laboratory procedures. The internal audit programme shall address all elements of the quality system, including the analysis. The audits have to be carried out by trained qualified personnel, which should be independent from the activity to be audited. Whenever nonconformances are found the laboratory shall take timely corrective actions according to section II.1.8. Control of Nonconforming Work, Corrective and Preventive Actions. The results of the internal audits shall be utilised as bases for annual reviews of the quality system documents. The activities of the laboratory must be audited internally once a year at least, and/or upon request of the management. The audit shall be organised, co-ordinated and monitored, according to an audit plan (*Figure 11*) containing the objectives and range of the audit proposed, indication and guidance to the auditors, date, estimated duration, a schedule and sectors to be audited. During the audit, the documents and activities audited should be recorded and the consistent evidences should be collected from the non-conformances found and corrective actions should be suggested. The final result of the audit shall contain the observations and classification of the non-conformances identified as major or minor non-conformances. The corrective and/or preventive actions proposed in the audit final report shall be assessed, selected, implemented and monitored until elimination of the nonconformances (ISO 17025 1999, MAPA 2001a).

Additional audits should be proposed and carried out whenever non-conformances raise doubts about the conformance of the Laboratory in regard of its own policies and procedures and of the standard ISO 17025 (1999).

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AUDIT PLAN - 2004	*Edited by Quality Manager* *Approved by: General Manager*

Objectives of Audits

To verify conformity of the Quality System and of the Laboratory procedures in regard of the Standard ISO/ 17025, Laboratory Quality Manual, and of the Standard Operational Procedures – SOP's.

Scope

Laboratory sectors: sample registration and preparation, glassware decontamination and cleaning, quality assurance, purchasing of services and supplies and mycotoxin analysis.

Indication and Guidance of Auditors

In compliance with the SOP Internal Audit, the Quality Manager shall orient the auditors.

Date, Estimated Duration and sectors to be Audited

Audit Nº	*Section*	*Participants*	*Month*
01-2004	*Purchasing of services and supplies*	*Person in charge of the sector* *Laboratory auditor*	*May*
02-2004	*Quality Assurance*	*Quality Manager* *Laboratory auditor or hired auditor*	*June*
03-2004	*Glassware decontamination and cleaning*	*Person in charge of the sector* *Laboratory auditor*	*July*
04-2004	*Sample registration and preparation*	*Person in charge of the sector* *Laboratory auditor*	*August*
05-2004	*Mycotoxins*	*Technical manager* *Laboratory auditor*	*September*

Chronogram:

To be defined 15 days prior to the predicted date of the Audit, and to be notified by means of written internal notice, to be signed by the auditors, the audited party, managers and personnel in charge of the audited sectors, as well as the quality manager and general manager.

Figure 11: Example of LACQSA Audit Plan

II.1.10. MANAGEMENT REVIEW

A management review should be made periodically, for evaluation of the documentation, organisation and management of the Laboratory, to identify the modifications and improvements needed, taking into consideration changes in the number and type of analyses carried out.

The following documents can be serve as bases for the management review:

- Internal audit reports;
- Complaint records and evaluation of customers;
- Non-conformance records;
- Results of internal quality control and inter-laboratory controls;
- Personnel training records;
- New project proposals.

The management review shall be registered, duly signed by the participants and must contain the dates of meetings, the subjects treated, the determinations and periods of time for implementation, which shall be verified afterwards. In view of the necessity of corrections, improvements or adequacy of the Laboratory documentation and procedures, the technical and quality management should settle a meeting, assess critically the question and propose the alterations needed. Other staff members can be invited to participate in the critical analyses, taking into consideration the content of the activities analysed. Those critical analyses should be registered and filed. The implementation of the modifications determined in the management review and critical analyses should be followed up (ISO 17025 1999, MAPA 2001a).

II.2. COMPLIANCE WITH TECHNICAL REQUIREMENTS

II.2.1. PERSONNEL

According to ISO 17025, the laboratory management shall:

- Ensure the competency of all who operate specific equipment, who perform tests, evaluate results and sign analysis reports;
- Provide appropriate supervision when using staff who are undergoing training;
- Qualify personnel performing specific tasks on the basis of appropriate instruction, training, experience and demonstrated skills, as required;
- Formulate the goals with respect to instruction and skills of the laboratory personnel;
- Have a policy and procedures for identifying training needs and providing training of personnel;
- Maintain current job descriptions for managerial, technical and key support personnel involved in tests;
- Authorise specific personnel to perform particular types of tests, issue analysis reports, give opinions and interpretations and operate particular types of equipment;
- Maintain records of relevant competence, educational and professional qualifications, training, skills and experience of all technical personnel. This information shall be readily available and shall include the date on which the authorisation or competence is confirmed and the criteria on which the authorisation is based and the confirming authority (*Figures 12* to *15*).

The analyst under training process can only be authorised to carry out a method after being capable to determine the toxin in a specific matrix with results within the criteria of acceptability defined by the Laboratory. The level and extent of training required shall be defined based on the internal *curriculum* of the personnel and on previous experience. The qualification of technicians on methods of performing mycotoxin analysis may comprise the following phases:

- Application of mycotoxin standard solutions on a plate or injection in a liquid chromatograph, with evaluation of the coefficient and parameters $\underline{a}$ and $\underline{b}$ of the calibration curve ($y=ax+b$), and repeatability of the areas obtained;
- Extraction of spiked samples in different levels (non blind test), and reference or spiked samples (blind test), in replicate, with evaluation of the recovery rate (%R), repeatability, and reproducibility (%RSD) obtained.

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Registration form ***Title:*** *Internal Curriculum*	*Page: X of Y* *Edited by: XX* *Approved by: XX*

Name:_____

Signature:_____

Date of entry at LACQSA:_____

Function:_____

Qualifications

Qualification	*Subject*	*Awarded by*	*Period*

Training Courses/Seminars/Workshops/Congresses

Date/Duration	*Title*	*Institution*	*Signature*

Publications

Date	*Title*	*Journal/Book*	*Signature*

Notes:_____

_____

_____

Figure 12: Example of internal *curriculum* of LACQSA technicians

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Registration form ***Title:*** *Internal Training on SOPs and IUs*	*Page: X of Y* *Edited by: XX* *Approved by: XX*

Name:

SOP or IU Code	*Ed*	*Rev*	*Date*	*Training Responsible Signature*	*Trainee Signature*	*RT Signature*	*Ed*	*Rev*	*Date*	*Training Responsible Signature*	*Trainee Signature*	*RT Signature*

Figure 13: Example of Internal Training form for LACQSA personnel

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Registration form ***Title:*** *Training and authorisation*	*Page: X of Y* *Edited by: XX* *Approved by: XX*

Name of trainee: ***Date:***

Responsible for training course:

Procedure:

Description of activities:

Approval by the technician in charge of training:

_____

Technician in charge of Training

Statement

I declare having received the above stated training and consider myself capable to perform the procedures as herein instructed.

_____

Trainee

Certificate *(to be filled when the trainee is authorised to perform the procedure)*

I, hereby, authorise the aforesaid trainee to perform the procedures described above and, substantiating the results obtained in the training course and/or qualification process, I certify that the supra qualified trainee is satisfactorily capable.

Date: ____/____/____

Renewal 1 ____/____/____ *Renewal 2* ____/____/____ *Renewal 3* ____/____/____

_____

Responsible Technician

Notes:_____

_____

_____

_____

Figure 14: Example of training form and authorisation of technicians on LACQSA's methods of analysis

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Registration form ***Title:*** *Training of technicians on methods of ochratoxin A analysis*	*Page: X of Y* *Edited by: XX* *Approved by: XX*

Matrix:
SOP(nº/ed./rev.): ***Period:***
Standard concentration (µg/mL):

Non blind samples:

Replicates	*Theoretical contamination level (µg/kg)*	*Contamination level determined (µg/kg)*	*Recovery (%)*	*Average Recovery (%)*	*RSD (%)*
1 *2* *3*					
1 *2* *3*					
1 *2* *3*					

Replicates	*Theoretical contamination level (µg/kg)*	*Contamination level determined (µg/kg)*	*Recovery (%)*	*Average Recovery (%)*	*RSD (%)*
1 *2* *3*					
1 *2* *3*					
1 *2* *3*					

Blind samples:

Replicates	*Theoretical contamination level (µg/kg)*	*Contamination level determined (µg/kg)*	*Recovery (%)*	*Average Recovery %)*	*RSD (%)*
1 *2*					

Replicates	*Theoretical contamination level (µg/kg)*	*Contamination level determined (µg/kg)*	*Recovery (%)*	*Average Recovery (%)*	*RSD (%)*
1 *2*					

Note: annex raw data, chromatograms, and calibration curve.

Technician ***Technician in charge of Training***

Figure 15: Example of registration form for training of LACQSA's technicians on ochratoxin A nalysis

II.2.2. ACCOMMODATION AND ENVIRONMENTAL CONDITIONS

The laboratory shall:

- Ensure that the facilities contribute for the correct performance of tests;
- Ensure that the environment does not invalidate the results or adversely affect the required quality of any measurement;
- Monitor, control and record environmental conditions as required by relevant specifications or where they might influence the quality of the results. Areas with incompatible activities shall be effectively separated, such as decontamination of glassware, sample preparation and analysis;
- Control the access to and use of areas affecting the quality of the tests, considering the hazard to health and safety of the personnel and assurance of reliability of the data generated, documents and rights of property of the Laboratory. All Laboratory sections should have identification signs and warnings on obligation of the use of individual protection equipment ("IPE"), that must be utilised before access is allowed. Access should be restricted to the Laboratory personnel involved in the activity developed at the location, or duly accompanied (*Figure 16*);
- Be equipped with security items. The staff involved in the stages of sample preparation and analysis must wear individual protection equipment, specific to each activity developed (*Figure 16*);
- Ensure adequate cleaning of the installations aiming at good performance of the activities. The waste material comprising a separate collection of wastes should be disposed separately according to their nature. The wastes from analyses and sample preparation should be treated as contaminated material, i.e., all solid materials or residues from analysis should be duly decontaminated prior to their disposal and/or incineration according to the country's environmental regulation (ISO 17025 1999, MAPA 2001a).

Figure 16: (a and b) Individual protection equipment (IPE); (c and d) LACQSA accommodation

II.2.3. METHODS OF ANALYSIS AND VALIDATION

According to ISO 17025 (1999), the laboratory shall use appropriate methods and procedures for all tests and, where appropriate, an estimation of the measurement uncertainty as well as statistical techniques for analysis of test data. All instructions, standards, manuals and reference data relevant to the work of the laboratory shall be maintained current and made readily available to personnel. Any deviation from test methods and procedures shall occur only if such deviation has been documented, technically justified, authorised and accepted by the customer.

II.2.3.1. SELECTION OF A METHOD

The laboratory shall use analytical methods which meet the needs of the customer and which are appropriate for the tests it applies, preferably those published as international, regional or national standards, or by reputable technical organisations, or in relevant scientific texts or journals. The methods adopted by the laboratory may also be used if they are fit for the purpose and if they are validated. The laboratory shall validate non-standardised methods, laboratory developed methods, standardised methods used outside their intended range and amplification of standardised methods to confirm that the methods are appropriate for the intended use. The validation shall be as extensive as necessary to meet the needs in a given application. The laboratory shall record the results obtained, the procedure used for the validation, and a statement as to whether the method is appropriate for the intended use (ISO 17025 1999).

The methods adopted by the Laboratory should be previously optimised and validated internally, taking into account the compliance with some criteria as shown in ***Table I*** (CEN 1999, MAPA 2001a). Analytical methods used by enforcement laboratories for implementation of legislation must be subject to international validation procedures, in order to show that the method produces reliable results (Gilbert and Anklan 2002).

Table I: Method's Performance Criteria for the Ochratoxin A Analytical Methods adopted by CEN (1999)

Level (µg/kg)	Recovery (%)	RSD_r	RSD_R
< 1	50 to 120	40	60
1 – 10	70 to 110	20	30

II.2.3.2. METHOD VALIDATION

The International Organisation for Standardisation (ISO 8402) defines validation as "confirmation by examination and provision of objective evidence that the particular requirements for a specified intended use are fulfilled". For analytical methods, this includes the establishment of performance characteristics, determining what influences may cause them to change and demonstrating

that the method is "fit for purpose" (EURACHEM/CITAC 1998).

The influences of the matrix and of procedures of extraction, clean up, detection and quantification on the efficiency of the method should be evaluated and determined. The validation steps should comprise: calibration using reference standard or reference materials, comparison of results achieved with other methods, interlaboratory comparisons, systematic assessment of the factors influencing the result, assessment of the uncertainty of the results based on scientific understanding of the theoretical principles of the method and practical experience (Boenke 1998, LACQSA 2001, Anklan 2002).

Method validation has to be a planned activity in a study plan having a minimum content involving the following items (ISO 1999, MAPA 2001a):

- Introduction;
- Objectives of study (scope);
- Description of the materials (matrix, mycotoxin, equipment, solutions, reagents and reference materials);
- Description of the procedure (including affixing of identification marks, handling, transportation, storing and preparation of samples, checks to be made before the work is started, checking that the equipment is working properly and, calibration and adjustment of the equipment before each use);
- Development and performance of tests;
- Original observations (method of recording);
- Analysis of data (criteria and/or requirements for approval/rejection, uncertainty or procedure for estimating uncertainty);
- Technical staff;
- Schedule;
- Bibliographic references.

During validation, the characteristics of the method should be determined in terms of (CODEX 2002, ISO 5725 1994):

- *Accuracy*: the closeness of agreement between the reported result and the accepted reference value (certified reference materials);
- *Applicability*: the analytes, matrices, and concentrations for which a method of analysis may be used satisfactorily to determine compliance with a CODEX standard;
- *Detection/determination limits*: detection limit is defined as field blank + 3σ, where σ is the standard deviation of the field blank value signal and determination limit is defined as field blank + 6σ or 10σ (IUPAC 1995). The determination limit is strictly the lowest concentration of analyte that can be determined with an acceptable level of repeatability precision and trueness;

- *Linearity*: the ability of a method of analysis, within a certain range, to provide an instrumental response or results proportional to the quality of analyte to be determined in the laboratory sample;
- *Precision*: repeatability intra-laboratory (within laboratory), reproducibility inter-laboratory (within laboratory and between laboratories): the closeness of agreement between independent test results obtained under stipulated conditions (ISO 3534-1). Repeatability: closeness of agreement between the results of successive measurements of the same measurand carried out in the same conditions of measurement. Reproducibility: closeness of agreement between the results of successive measurements of the same measurand carried out in reproducibility conditions: same method on identical test items in different laboratories with different operators using different equipment;
- *Recovery*: proportion of the amount of analyte present or added to the test material which is extracted and presented for measurement;
- *Selectivity*: is the extent to which a method can determine particular analyte(s) in mixtures or matrices without interferences from other components;
- *Sensitivity*: change in the response divided by the corresponding change in the concentration of a standard (calibration) curve; i.e., the slope, s_1, of the analytical calibration curve.

II.2.3.3. ESTIMATION OF MEASUREMENT UNCERTAINTY

The laboratory shall have and apply a procedure to estimate the measurement uncertainty. The laboratory shall identify all the components of uncertainty and make the best possible estimation, and ensure that the form of reporting does not give an exaggerated impression of accuracy (ISO 17025 1999).

The International Vocabulary of Basic and General Terms in Metrology (1993) defines uncertainty as "a parameter associated with the result of a measurement, that characterises the dispersion of the values that could reasonable be attributed to the measurand".

According to EURACHEM/CITAC (2000b) and ISO Guide (1993), measurement uncertainty comprises many components that may be evaluated from the statistical distribution of the results of a series of measurements and can be characterised by standards deviations. The other components, which also can be characterised by standard deviations, are evaluated from assumed probability distributions based on experience or other information.

CODEX (2003) discurss that the measurement uncertainty of an analytical result may be estimated in a number of procedures, notably those described by ISO Guide (1993) and EURACHEM/CITAC (2000). These documents describe procedures for measurement uncertainty based on a component-by-component approach, method validation data (reproducibility determined during validation of analytical methods), internal quality control data (treatment of the recovery data, when the method becomes a routine method of the Laboratory) and proficiency test data. An estimation of the

measurement uncertainty using the ISO component-by-component approach is not necessary if other forms of data are available and used to estimate the uncertainty. In many cases the overall uncertainty can be determined by an inter-laboratory (collaborative) study made by a number of laboratories and a number of matrices by IUPAC/ISO/AOAC/International or by the ISO 5725 Protocols.

In the Report of the Standing Committee on the Food Chain and Animal Health (FSA 2003) the following procedures were proposed to aid the estimation of the measurement uncertainty :

- ISO guide to the expression of uncertainty in measurement;
- EURACHEM Guide to quantifying uncertainty in analytical measurement:

  A. component-by-component approach;

  B. use of collaborative trial data;
- Use of collaborative trial: data - ISO 5725 critical differences;
- Draft ISO TS 21748 – Guide to the use of repeatability, reproducibility and trueness estimates in measurement uncertainty estimation;
- Concept settled by the commission decision 2002/657/EC Implementing the council directive 96/23/EC, concerning the performance of analytical methods and interpretation of results;
- AOAC INTERNATIONAL approach;
- Internal quality control approach;
- NMKL (Nordic Committee on Food Analysis) approach.

II.2.4 EQUIPMENT

"Equipment" shall be understood as the facilities utilised for analysis such as instruments, standard mass, thermometers, micro-pipettes and volumetric glassware including those utilised at processing of data and results (fax and computers) (MAPA 2001a).

The laboratory shall be furnished with all items of measurement and test equipment required for the correct performance of the tests. The laboratory shall have all instruction manuals required on the use and operation of the relevant equipment. All equipment used for tests having any significant effect on the accuracy or validity of test results should be calibrated before being put into service. The laboratory shall have a programme and procedure established for calibration of its equipment (ISO 17025 1999).

Upon receipt, the pieces of equipment shall be duly checked, identified (*Figure 17*), calibrated by a calibration laboratory capable to demonstrate competence, capacity for measurement and traceability to the International Unit System, or that has its performance evaluated, before the equipment is available for use, according to the equipment plan. Only qualified personnel, aware of updated instructions of use are authorised to operate the equipment. Preventive maintenance programs, assessment of performance and of calibration need to be predicted and established for key quantities or values of the instruments where these properties have any significant effect on the results. The Equipment Plan (*Figure 18)* shall contain data for preventive maintenance, calibration and performance evaluation. Corrective maintenance programs shall be carried out when necessary. Whenever any alteration or problem affecting or which may affect the operation of an equipment is noticed, or when results of performance evaluation out of the criteria of acceptability previously defined are observed, these should be clearly identified as out of use, except in situations properly justified. The maintenance of the equipment should be readily available, and must be done only by the authorized technicians. After release of the equipment by the technician and/or entity that renders maintenance services, the equipment should be checked and, if it is found in perfect condition for operation, the equipment is placed back into use. *Figure* 19 illustrates procedures for equipment policy. Performance evaluation (checks) and calibrations are carried out within the frequency and necessity establishedas shown in ***Table II***, according to the procedures described in the respective SOP's. The results of these checks shall be utilised for establishing the level of confidence on the *status* of calibration of the equipment. Preventive maintenance should be carried out once a year at least, for equipment used in the analyses or which interferes in their results. The equipment used for sample preparation, analysis, and emission of results should have all related instructions of use and performance evaluation report available (when it is the case), in printed form, where the equipment is located (ISO 17025 1999, MAPA 2001a).

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Registration form ***Title:*** *Registration of equipment*	***Page:*** *x of y* ***Edited by:*** *XX* ***Approved by:*** *XX*

Equipment registration Nº

Equipment:

Brand:

Model:

Series Nº.:

Source:

Date of entry at Laboratory:

Registered by: ***Signature / Initials:***

Date of start of utilisation:

Date of withdrawal from use:

Location:

Notes:

_____
_____
_____
_____
_____
_____
_____
_____

Figure 17: Example of LACQSA Equipment Registration Form

MINISTRY OF AGRICULTURE, LIVESTOCK AND SUPPLY OF BRAZIL *LABORATORY FOR QUALITY CONTROL AND FOOD SAFETY - LACQSA/LAV-MG*									*Edition: XX* *Review: XX* *Date: Day/Month/Year* *Page: X of Y*				
EQUIPMENT PLAN									*Edited by: XX* *Approved by: XX*				
EQUIPMENT	***Register number***	*JAN (Date)*	*FEB (Date)*	*MAR (Date)*	*APR (Date)*	*MAY (Date)*	*JUN (Date)*	*JUL (Date)*	*AUG (Date)*	*SEP (Date)*	*OCT (Date)*	*NOV (Date)*	*DEC (Date)*
Vortex mixer	*030*				*PM*								
Scale	*003*	*PC*	*PC*	*PC*	*PC*	*PC*	*PC*	*PC*	*PC*	*PC*	*PM* *PC*	*C* *PC*	*PC*
	057	*PM* *PC*	*C* *PC*	*PC*	*PC*	*PC*	*PC*	*PC*	*PC*	*PC*	*PC*	*PC*	*PC*
Bath	*005*						*PM*						*PM*
Dough mixer	*195*		*PM*						*PM*				
Cool chamber	*215*	*PM* *PC*	*PC*	*PM* *PC*	*PC*	*PM* *PC*	*PC*	*PM* *PC*	*PC*	*PM* *PC*	*PC*	*PM* *PC*	*PC*
LC apparatus	*212* *212-2*		*Cert*										
Densitometer ***Printer***	*009* *009-2*	*PC*	*PC*	*PC*	*Cert* *PC*	*PC*	*PC*	*PC*	*PC*	*PC*	*PC*	*PC*	*PC*
Spectrophotometer	*010*	*PC*	*PC*	*PC*	*PC*	*Cert* *PC*	*PC*	*PC*	*PC*	*PC*	*PC*	*PC*	*PC*

PM: preventive maintenance, PC: performance check, C: Calibration, Cert: certification

Figure 18: Example of LACQSA Equipment Plan

Table II: Example of frequency of performance evaluations (checks) and calibration of some pieces of equipment.

EQUIPMENT	PERFORMANCE EVALUATION (CHECKS)*	CALIBRATION*
	ASPECT EVALUATED	
SCALES	Determination of standard mass	YES
WATER BATHS and OVENS	Accuracy and repeatability between triplicates of temperature readings using a calibrated thermometer	NO
SPECTROPHOTOMETER	Factor of correction determined by values of absorbance of solutions of potassium dichromate	YES
FREEZER, COOL CHAMBER & FRIDGE	Acceptable range of temperatures readings using a calibrated thermometer	NO
MICRO-PIPETTES	Accuracy and repeatability between 10 determinations of mass of volumes measured	YES
STANDARD MASS	NO	YES
THERMOMETERS	Comparison with reference thermometer	YES
GLASSWARE	Weighing (comparison with calibration rated value)	YES

*The frequency of internal checks and of calibrations should be defined based on the previous results obtained from the checks and calibrations and on evaluation of the amount of error interference exerted by the equipment on the analytical result to be issued.

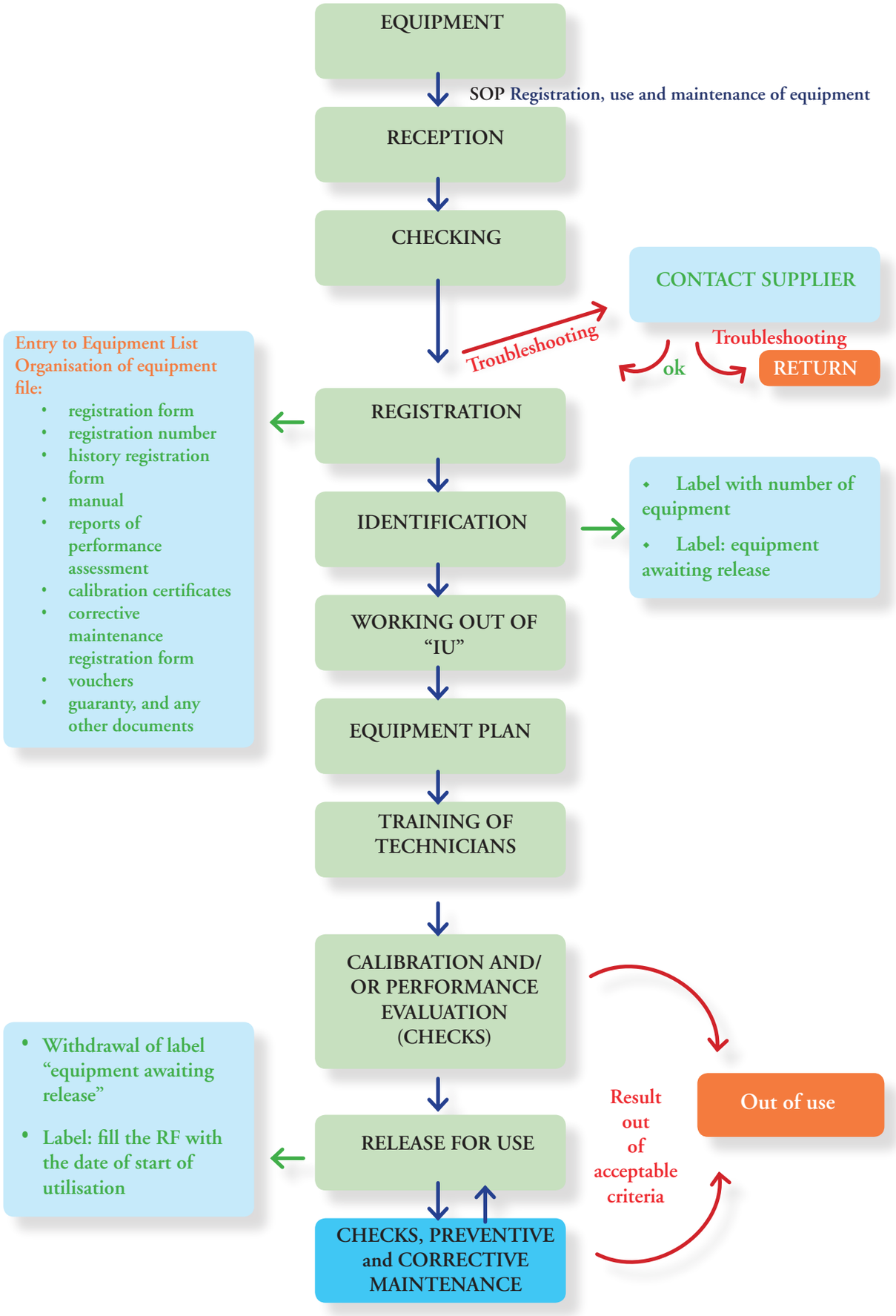

Figure 19: General LACQSA procedures established for equipment

II.2.5. MEASUREMENT TRACEABILITY

II.2.5.1. SPECIFIC REQUIREMENTS

Traceability of measurement shall be assured by the use of calibration services made by laboratories capable to demonstrate competence, measurement capability and traceability to the SI (Système International).

Wherever traceability to SI is not possible, the following shall be applied: the use of suitable certified reference materials, mutual-consent standards or participation in a suitable programme of interlaboratory comparisons or proficiency testing. The key elements in the establishment of traceability are (EURACHEM/CITAC 2003):

- To specify the measurand, the scope of measurements and the required uncertainty;
- To choose a suitable method of estimating the value, that is, a measurement procedure with associated calculation – an equation – and measurement conditions;
- To demonstrate, through validation, that the calculation and measurement conditions include all the "influence quantities" that significantly affect the result, or the value assigned to a standard;
- To identify the relative importance of each influence quantity;
- To choose and apply appropriate reference standards;
- To estimate the uncertainty.

II.2.5.2. STANDARDS AND REFERENCE MATERIALS

The laboratory shall have a programme and procedure for calibration of its reference standards. The standards and reference materials, upon receipt, have to be checked, registered, labelled, handled, stored so as to assure their traceability and integrity, and their stocks have to be controlled. Information like sequential number, entry date, substance, source, batch number, pureness and quantity should be recorded (ISO 17025 1999, MAPA 2001a).

The mycotoxin standard solutions should be prepared from solid or liquid standards. Preparation and standardisation of these solutions should be recorded and controlled. Periodically, these solutions must be submitted to re-standardisation for verification of concentration, and the variability criteria between the standardisation should be established (MAPA 2001a, AOAC 2000).

When a certified reference material is not available it may be necessary to prepare an in-house reference material. The laboratories can prepare their own reference material (in-house reference material) and determine an assigned value through a careful analysis by using a naturally contaminated sample, according to the International Harmonised Protocol for Proficiency Testing of (Chemical) Analytical Laboratories (Thompsom and Wood 1993), and IUPAC Harmonised Protocol for Design, Conduct and Interpretation of Collaborative Studies (IUPAC 1995).

Figure 20: LACQSA in-house reference material

II.2.6. SAMPLING

The laboratory shall have a sampling plan and procedures for sampling when carrying out sampling for subsequent testing. If the quality of sampling is questionable, a "not reliable" statement shall be produced for the analytical data obtained, independently of the quality of the analytical method. Sampling is a defined procedure whereby a part of a substance, matrix, material or product is taken for testing a representative sample of the whole. Sampling plans may be random, systematic or sequential, and they may be undertaken to obtain quantitative or qualitative information, or to determine conformance or non-conformance with a specification (ISO 17025 1999).

II.2.7. HANDLING OF SAMPLES

The laboratory shall have procedures for reception, handling, protection, retention and disposal of samples, according to *Figure 22*.

II.2.7.1. RECEPTION

The laboratory shall establish procedures and conditions to receive and identify samples. The identification shall be preserved during the existence of the sample in the laboratory. The conditions of the samples should be monitored to prevent damage to the packing and, appropriate facilities should be guaranteed to prevent deterioration, loss or damage during storage, handling and preparation. In case of damage or any other abnormality, the sample should be disposed or replaced with another one, upon understanding with the customer. The sample should not be received if the packing is damaged or if the sample is deteriorated to the extent that the laboratory deems as unacceptable. The sample - duly identified and sealed - sent to laboratory shall be accompanied by the "Term of Remittance of Sample" (*Figure 21*) or a "Sampling Protocol" duly filled out according to laboratory specific instructions or a sampling protocol. In case the sample received is non-conforming with laboratory specific instruction or a sampling protocol, it shall be received upon agreement between the laboratory and the customer. Any and all observations or definitions related to the samples, other than those predicted in the laboratory procedure should be properly registered in an appropriate registration form field and signed by the person in charge and informed to the customer (ISO 17025 1999, MAPA 2001a).

Term of Remittance of Sample		
Sample code	***Entry date***	

Exclusive use of the Laboratory

Customer*

1.Name / Requesting Institution:		
2.Address / City / State:		
3.Zip code:	*4.Telephone:*	*5.Fax:*

Product Identification*

6.Product:	
7. ☐*Producer* ☐ *Storekeeper* ☐*Trader* ☐ *Importer* ☐*Exporter* ☐*Packer* *Name:*	
8. Coming from (city / State):	
9.Destination:	
10.Transportation:	*11. Crop:*
12. Lot size (kg or metric ton.):	
13. Lot nº:	*14. Date of collection:*
15. Size of sample sent to laboratory (kg):	
16. Storage place:	
17. Storage condition:	
18. Sampling protocol number:	*19. Sample nº:*
20. Seal number:	*21. Product registration nº:*
22. Sampling place (city / State):	
23. Analysis (es) requested:	
24.Nature of operation (objective of analysis): ☐ *Inspection* ☐*Survey* ☐*other:*	

The information above will be transcribed to the Analysis Report.

Notes:

Place and date *Signature*

Figure 21: Term of Remittance of Sample for determination of mycotoxins

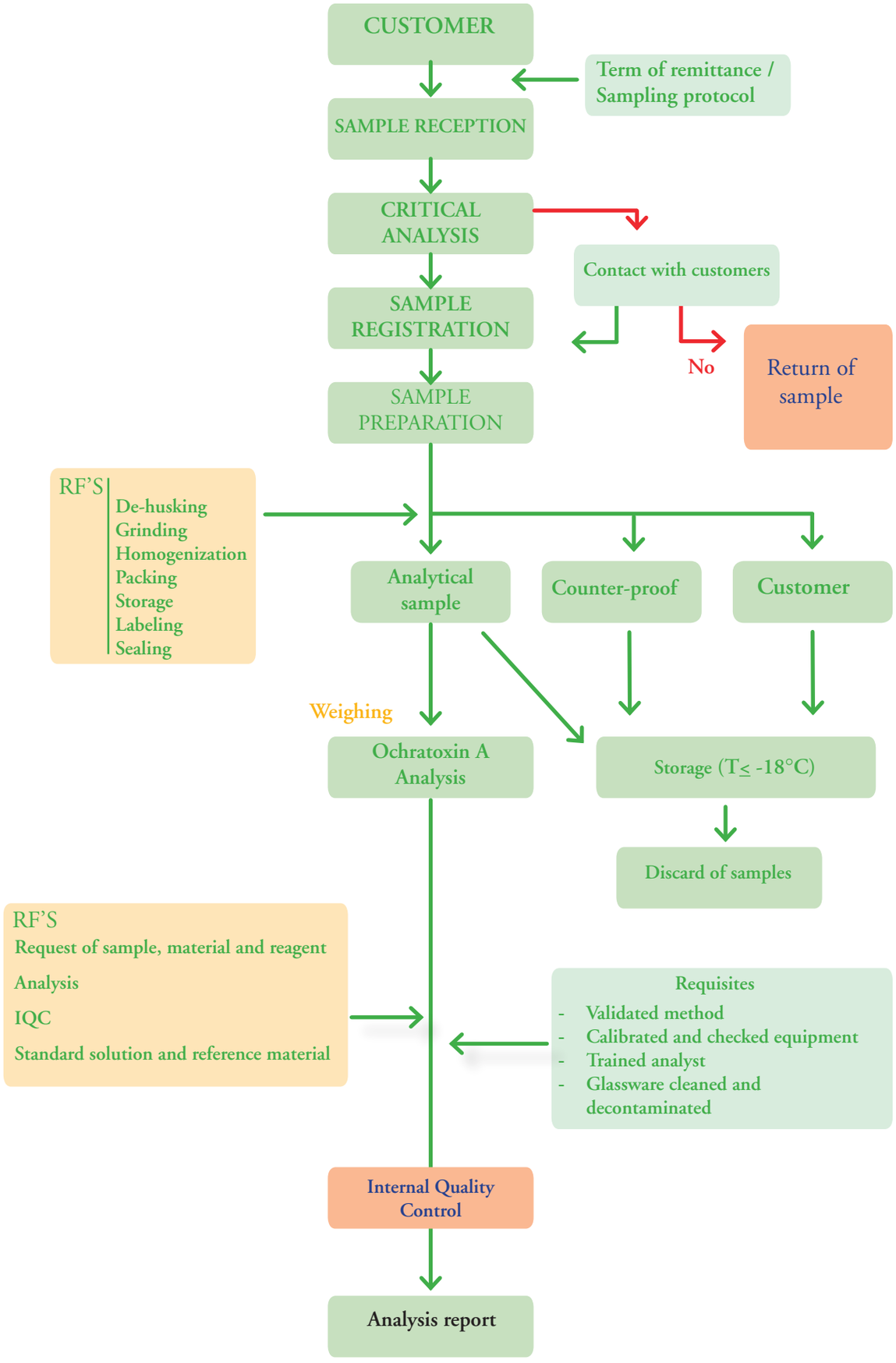

Figure 22: Flow chart of handling the test item from reception to analysis report

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Registration Form ***Title:*** *Registration of samples/analysis*	*Page: X of Y* *Edited by: XX* *Approved by: XX*

Sample code at LACQSA	*Matrix*	*Date of entry*	*Customer*	*Customer sample number*	*Mycotoxin*	*Result (µg/kg)*	*Date of start of analysis*	*Method (SOP/% R/LOD)*	*Analyst's name*	*Date of Report*	*Analysis report nº*	*Date of disposal*

Notes: _____
_____
_____

Figure 23: Example of sample registration form

II.2.7.2. REGISTRATION

The samples are recorded according to the information provided by the customer, contained in the "Term of Remittance of Samples" or "Sampling Protocol" (*Figure 21*). Recording aims at a unique identification of the sample, assuring its localisation and identification during the period of storage. The samples shall receive a unique code number to represent the year of their registration, origin and sequential entry number in the laboratory (MAPA 2001a).

II.2.7.3. PREPARATION AND STORAGE

The samples should be prepared aiming at assuring their homogeneity, representativeness in regard of the original samples received by the laboratory, integrity and preservation. The samples should be stored at a temperature $T \leq -18$ °C. The procedures for grinding and homogenisation should ensure the smallest particle size and greatest homogenisation possible, assuring a representative result of the sample analysed (MAPA 2001a). The laboratory, taking in account its facilities, must validate the grinding and homogenisation procedures.

II.2.7.4. RETENTION AND DISPOSAL

The laboratory must have proper facilities for storage and security to protect the condition and integrity of the sample and should define a period and procedures for safe disposal of samples, including all provisions necessary to protect the integrity of the laboratory. The samples should be disposed after a period determined by the laboratory from the date of the analysis report or result of analysis (MAPA 2001a).

II.2.8. ANALYTICAL QUALITY ASSURANCE

According to ISO 17025 (1999), the laboratory shall ensure the quality of results by monitoring test results. This monitoring shall be planned and reviewed and may include, but not be limited to, the following:

- Internal quality control schemes;
- Participation in interlaboratory comparison or proficiency testing programmes;
- Regular use of certified reference materials and/or internal quality control using secondary reference materials;
- Replicate tests or re-testing using the same or different methods.

The results obtained are tools for evaluation of the Laboratory performance, indicating the necessity of adoption of corrective actions in case these show themselves questionable or unsatisfactory (treatment of nonconforming work).

II.2.8.1. INTERNAL QUALITY CONTROL

Internal quality control (IQC) is an essential tool for ensuring that the data released are "fit for purpose" and enables monitoring the quality of the data on a run-by-run basis (Gilbert 2002). The basic approach to IQC involves the analysis of control materials alongside the test materials under examination. The outcome of the control analyses forms the basis of a decision regarding the acceptability of the test data (IUPAC 1995b).

Recovery tests shall be carried out at each batch of samples, employing control samples – spiked and/or naturally contaminated (in-house or certified reference materials) blind and non-blind to analysts, followed by blank samples (MAPA 2001b).

The results of analysis shall only be considered acceptable if the control samples meet the recovery criteria established by the laboratory. The criterion herein established is that adopted by the CEN – European Committee for Standardization: 50-120% and 70-110% for ochratoxin A contamination of <1 and 1-10 µg/kg, respectively (CEN 1999).

The internal quality control scheme can be seen through *Figure 24*.

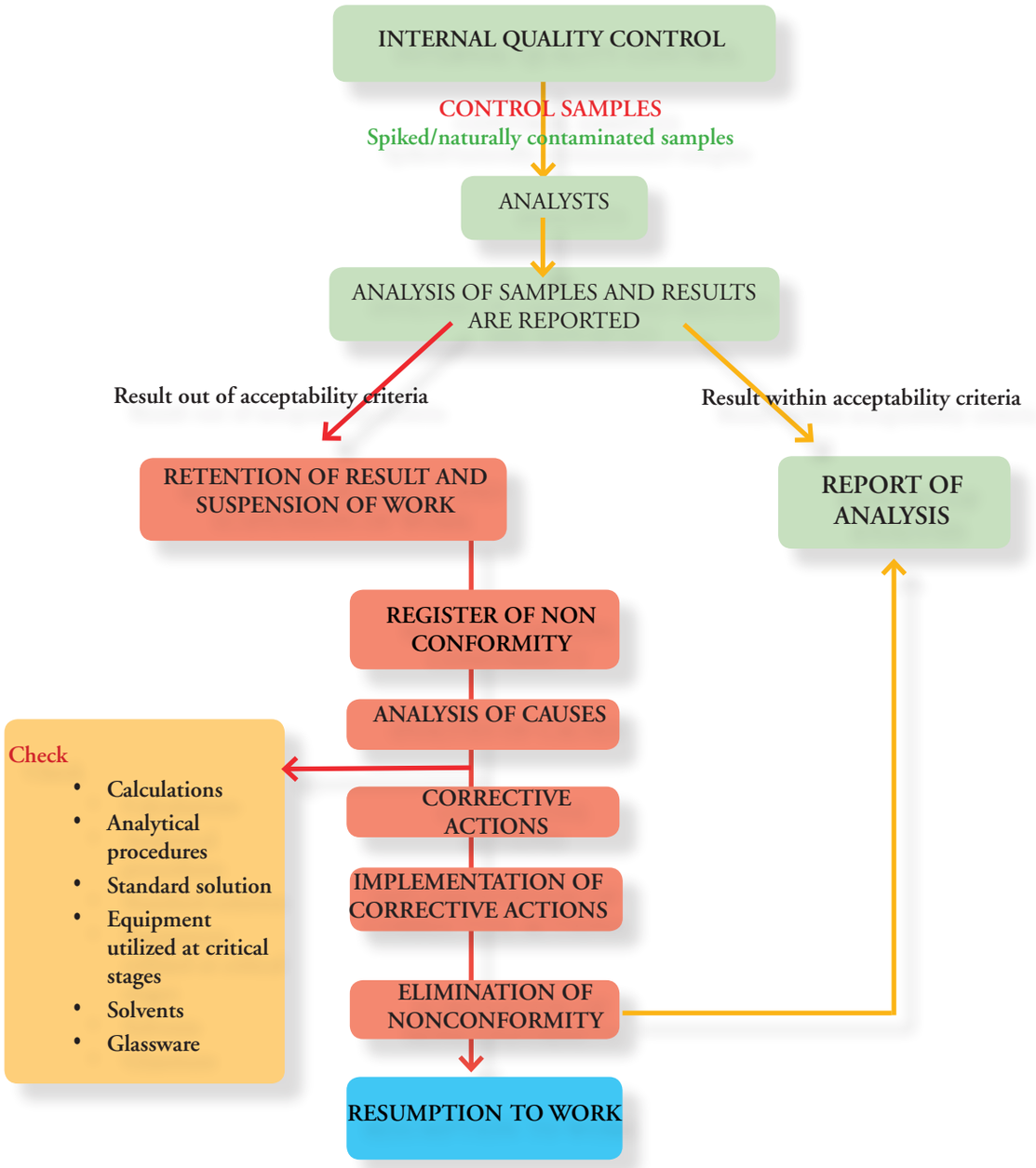

Figure 24: Internal quality control scheme

II.2.8.2. CONTROL CHARTING

Control charting is a useful tool in the internal quality control that makes possible the display of quality control of a given analytical process. A control chart is a graph of test results with respect to time or sequence of measurements, with limits drawn within which results are expected to lie. Their use is strongly suggested when a sample with a known standard deviation is run on a routine basis. The control charts must be set up when the process is in control: the analyst must be familiar with the method, must have explored the various sources of error and use a validated method. Shewart control charts are the easiest to construct, use, and interpret (Garfield *et al.* 2000) (*Figures 29* to *31).* In food analysis the Horwitz curve is sometimes used as a fitness for purpose criterion (IUPAC 1995b).

Preferentially, a well-characterized homogeneous reference material shall be used to set up the control charts (LACQSA 2001a, 2001b). Homogeneous material shall be prepared according to international harmonized protocols (Horwitz 1988, Thompson and Wood 1993, IUPAC 1995a, ISO 1997) (*Figures 27* and *28).*

The mean result and the standard deviation representing run-to-run variation shall be determined and the value of Xm ± s used to establish the control limits within the maximum predicted variability (Xm ± 2s – warning limit). The results within Xm ± 3s are considered out of control and demand that an action should be taken to solve the non-conformity (Gilbert 2002, IUPAC 1995b). Results from recovery tests can also be displayed in control charts where the maximum and minimum acceptable recovery is shown. Examples of control charts for naturally and spiked samples are given in *Figures 25* and *26*.

Figure 25: Example of Control chart of in-house material [naturally contaminated coffee sample, (4.99 + 0.58 µg/kg, n=20), n=78], SOP 039 ed. 03, rev. 02 (Immunoaffinity column clean-up and HPLC)

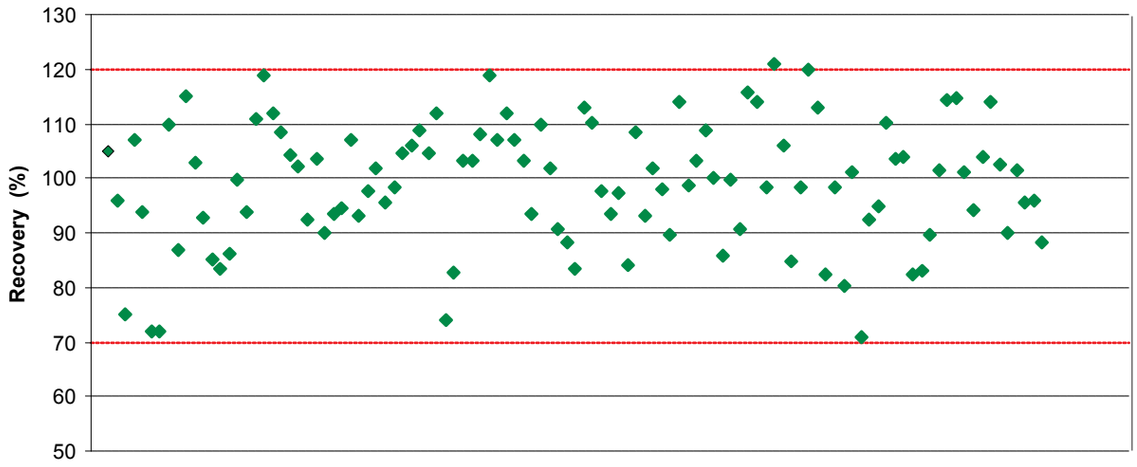

Figure 26: Example of Control chart for spiked coffee sample, n=109, mean recovery 99%, Relative Standard Deviation (internal reproducibility) of 11.4%, SOP 039 ed. 03, rev. 02 (Immunoaffinity column clean-up and HPLC)

MINISTRY OF AGRICULTURE, LIVESTOCK AND SUPPLY OF BRAZIL *LABORATORY FOR QUALITY CONTROL AND FOOD SAFETY - LACQSA/LAV-MG*	*Code: RF – XX* *Edition: XX* *Review: XX* *Date: Day/Month/Year*
Registration Form ***Title:*** *Test sample (blank or naturally contaminated)*	*Page: X of Y* *Edited by: XX* *Approved by: XX*

Test sample code	*SOP (ed./rev.)*	*Registration date*	*Matrix*	*Mycotoxin*	*Assigned value (µg/kg)*	*Standard deviation (s)*	*Date of disposal or of completion*
04BR001	*039 (03/02)*	*Dec,8th, 2003*	*Green coffee*	*Ochratoxin A*	*0,20*	*0,05*	
04NC002	*039 (03/02)*	*Dec,8th, 2003*	*Green coffee*	*Ochratoxin A*	*4,99*	*0,58*	

*Notes:*_____

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Figure 27: Registration Form: Test sample (blank or naturally contaminated)

MINISTRY OF AGRICULTURE, LIVESTOCK AND SUPPLY OF BRAZIL *LABORATORY FOR QUALITY CONTROL AND FOOD SAFETY - LACQSA/LAV-MG*	*Code: RF – XX* *Edition: XX* *Review: XX* *Date: Day/Month/Year*
Registration Form ***Title:*** *Reference materials (samples)*	*Page: X of Y* *Edited by: XX* *Approved by: XX*

Sample code	*Entry Date*	*Matrix*	*Source*	*Reference/ Number*	*Mycotoxin*	*Assigned value*	*Purpose*	*Data/Signature*
04RM001	*8th March, , 2004*	*Green coffee*	*Supplier X*	*TO 08440*	*Ochratoxin A*	*5,00*		

*Notes:*_____

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Figure 28: Registration Form: Reference materials (samples)

MINISTRY OF AGRICULTURE, LIVESTOCK AND SUPPLY OF BRAZIL *LABORATORY FOR QUALITY CONTROL AND FOOD SAFETY - LACQSA/LAV-MG*	*Code: RF – XX* *Edition: XX* *Review: XX* *Date: Day/Month/Year*
Registration Form ***Title:*** *Control chart – reference material*	*Page: X of Y* *Edited by: XX* *Approved by: XX*

Test sample code: 04NC002 **Matrix: Green coffee** **Assigned value (Xm): 4.99 µg/kg** **s (standard deviation): 0,58 µg/kg**

SOP (ed/rev): 039 (03/02) **Mycotoxin: Ochratoxin A** **Year: 2004**

Control nº	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Date (day/ month)	01/ 15	01/ 20																												
Analyst																														
Result (µg/kg)	4,5	5,1																												

+3s = 6,73																														
Warning limit																														
+2s = 6,15																														
Action limit																														
+ s = 5,57																														
Assigned value = 4,99		x																												
- s = 4,41	x																													
Action limit																														
- 2s = 3,83																														
Warning limit																														
- 3s = 3,25																														

*Notes:*_____

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Figure 29: Registration Form: Control chart – reference material

MINISTRY OF AGRICULTURE, LIVESTOCK AND SUPPLY OF BRAZIL *LABORATORY FOR QUALITY CONTROL AND FOOD SAFETY - LACQSA/LAV-MG*	*Code: RF – XX* *Edition: XX* *Review: XX* *Date: Day/Month/Year*
Registration Form ***Title:*** *Control chart – spiked sample*	*Page: X of Y* *Edited by: XX* *Approved by: XX*

Matrix: Green coffee ***Mycotoxin: ochratoxin A*** ***SOP (ed/rev): 039 (03/02) Year: 2004***

Control nº	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*	*19*	*20*	*21*	*22*	*23*	*24*	*25*
Date (day/ month)	*01/ 16*	*01/ 19*																							
Analyst																									
Test sample code	*04B R00 1*	*04B R00 1*																							
Recovery (%)	*89*	*100*																							

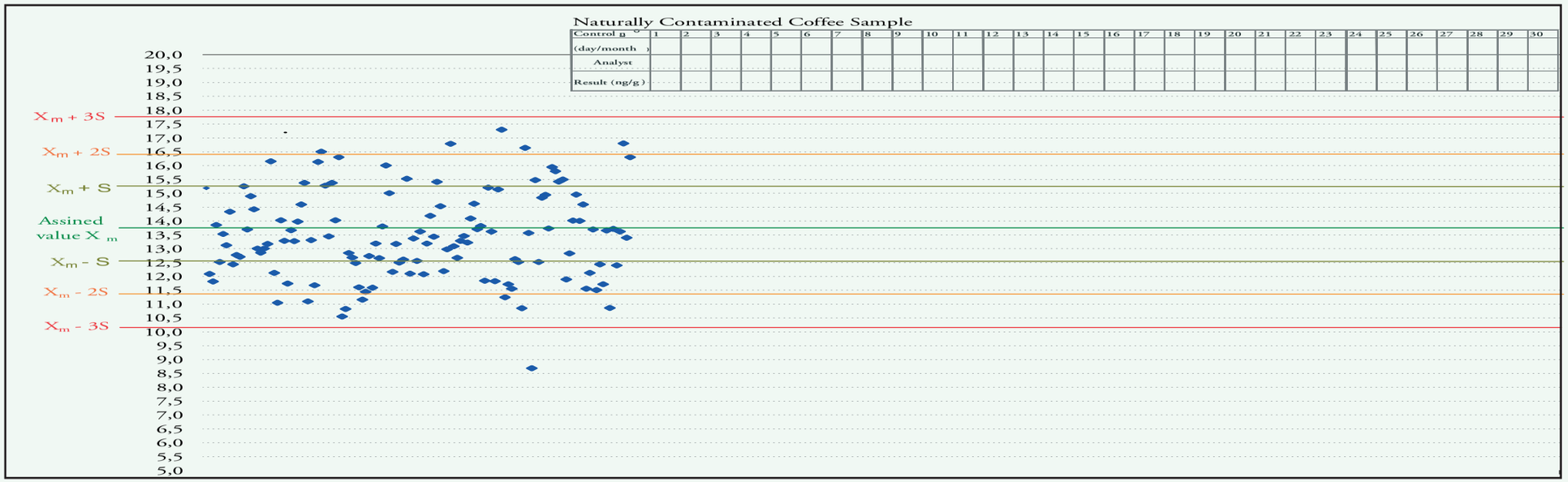
Recovery (%)																									
140																									
130																									
120																									
110																									
100		*x*																							
90	*x*																								
80																									
70																									
60																									
50																									

*Notes:*_____

Figure 30: Registration Form: Control chart – spiked sample

MINISTRY OF AGRICULTURE, LIVESTOCK AND SUPPLY OF BRAZIL *LABORATORY FOR QUALITY CONTROL AND FOOD SAFETY - LACQSA/LAV-MG*	*Code: RF – XX* *Edition: XX* *Review: XX* *Date: Day/Month/Year*
Registration Form ***Title:*** *Control chart – Naturally contaminated sample (reference material)*	*Page: X of Y* *Edited by: XX* *Approved by: XX*

Test sample code: 03NC013 **Matrix:** Green coffee **Assigned value (Xm):** 13,46 µg/kg **s (standard deviation):** 1,18 µg/kg
SOP (ed/rev): 039 (03/02) **Mycotoxin:** Ochratoxin A **Year:** 2003



*Notes:*_____

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Figure 31: Registration Form: Control chart – Naturally contaminated sample (reference material)

II.2.8.3. PROFICIENCY TESTING PROGRAMMES

The requirements for establishing and running proficiency testing schemes are stipulated in an ISO/IUPAC/AOAC International Harmonised Protocol and ISO Guide 43 (1997). There are a number of commercial schemes which are run on a regular and systematic basis. Proficiency Testing is a tool for continuous assessment of the ability of a laboratory to produce accurate and reliable results. When taking part in a Proficiency Testing Scheme, the laboratory should choose the toxin and matrix routinely tested in the laboratory. If the desired matrix is not available a similar one should be done. The participant laboratories shall receive samples at regular intervals, use a methodology in place, they should report the results to the organisers and are then informed of an assessment of their performance (*Figure 33*). The participant laboratories receive a report with useful indications about the overall performance of all participants and information on the effectiveness of the methods of analysis employed (FAPAS 2002). The laboratories identifications shall be kept confidential (EURACHEM 2000a).

Figure 32 shows the schematic treatment given by the Laboratory to the proficiency testing results. When the lz-scorel > 2, a non-conformance is recorded as shown in the flow chart in *Figure 10*.

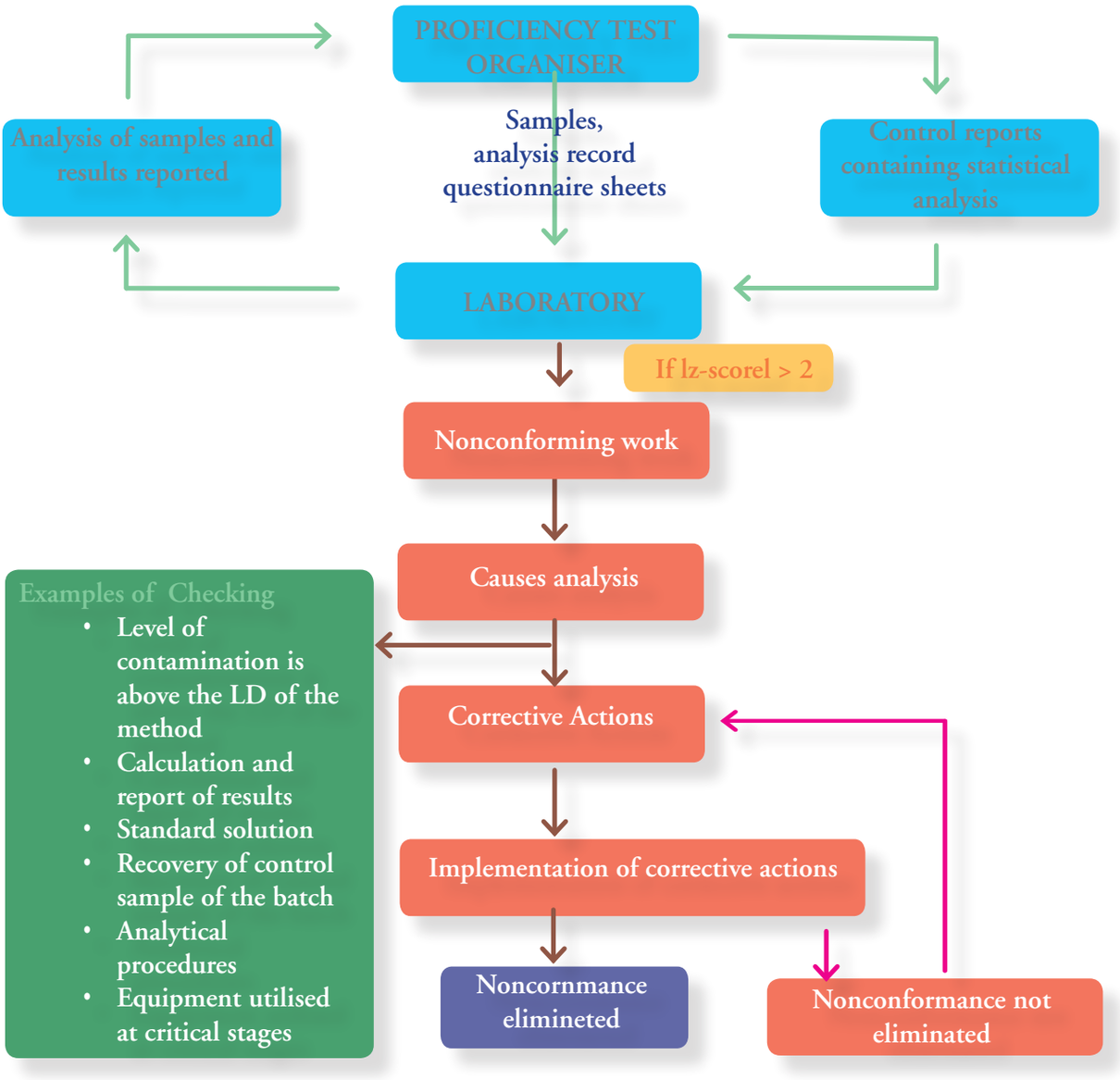


Figure 32: Flow chart of treatment of proficiency testing results

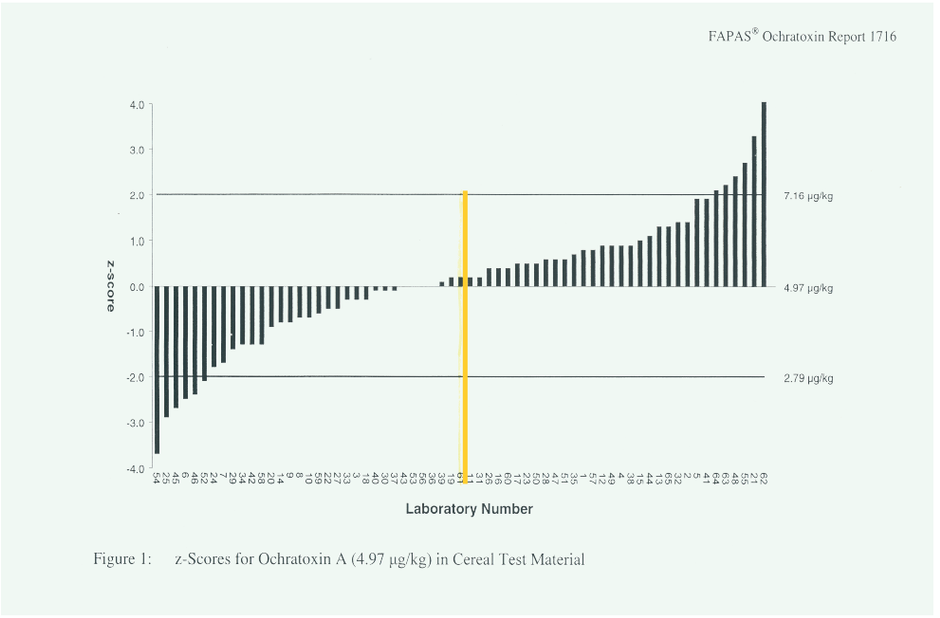


Figure 1: z-Scores for Ochratoxin A (4.97 µg/kg) in Cereal Test Material

Figure 33: Proficiency testing scheme result (FAPAS 2002)

II.2.9. REPORTING THE RESULTS

The results of each analysis carried out by the laboratory shall be reported accurately, clearly, unambiguously and objectively, and shall include all the information requested by the customer and necessary for the interpretation of the analysis results, in accordance with specific instructions in the test methods (MAPA 2001a).

II.2.9.1. ACCEPTABILITY OF RESULTS

The analytical results shall only be reported if the internal quality control results and the parameters of the calibration curves, as correlation coefficient (r^2), *a* and *b* values in the equation $ax + b$ meet the criteria established as acceptable by the Laboratory. In case the results do not meet the criteria established, the analysis should be repeated and the non-conformities detected (see item II.2.8 Analytical Quality Assurance).

The analytical raw data shall be written down initially in the analysis follow up registration forms, filed along with the respective records and chromatograms, and the results of analysis are recorded in the logbook or computerised system.

Important aspects shall be considered when reporting an analytical result, such as:

- The number of significant figures taken into account when reporting and interpreting them against statutory limits;
- The treatment of analytical variability (measurement uncertainty) in the interpretation of a specification;
- The use of recovery values for correction of analytical results.

The results shall be released in the form of "Analysis Report" as exampled in the *Figure 34,* with a unique identification (see registration form), containing the information supplied by the customer in the "Term of Remittance of Sample". These shall be checked and signed and forwarded to the customer. Copies of the analyses reports shall be maintained in a proper file and kept in safety. No form of correction shall be accepted on reports and results of analysis. If needed, the amends should be made only under form of a new document, bringing clearly the statement: "Errata to the Report of analysis ex. nr. 00/0X" and this document cancels and replaces the Report ex nr. 00/0Y. The Analysis Report must contain, at least the information below (MAPA, 2001a):

1) Title: Test Report
2) Number of report (unique identification)
3) Sample code at laboratory
4) Name and address of the customer
5) Identification of Product:

- Product;
- Producer/Storekeeper/Dealer/Importer/Exporter/Packer;
- Origin (city/State);
- Destination;
- Transportation;
- Crop (year);
- Batch size (kg or metric ton.);
- Lot Nº ;
- Incremental size;
- Date of collection;
- Sample size received by Laboratory (kg);
- Location of storage;
- Storage condition;
- Collection term Nº;
- Sample Nº;
- Seal Nº ;
- Product registration Nº ;
- Location of collection (city / State);
- Analysis (es) requested;
- Nature of operation (objective of analysis): fiscalization / inspection / research;
- Date of receipt of sample.

6) Data Regarding Analysis:

- Results;
- Method of analysis;
- Limit of detection/determination;
- Measurement uncertainty;
- Recovery;
- Date of Analysis.

7) Additional data:

- Related regulation;
- Date of report;
- Laboratory statement: Example: The result(s) of this (these) analysis(es) is (are) restricted and applies only to samples sent to the laboratory by the customer. The Laboratory does (not) perform sampling. The document must not be reproduced partially;
- Name and address of Laboratory;
- Number of pages (*x* of *y*).

8) Name(s) and Signature(s):

- Technical Manager;
- Analyst Technician.

OCHRATOXIN A ANALYSIS REPORT Nº XXX / XX (unique identification)

		Sample code at Laboratory:
1. Name / Agency / Requesting Institution:		
2. Address / City / State:		
3. Zip code:	*4. Telephone:*	*5. Fax:*

Product Identification

6. Product:	
7. ☐ Producer ☐ Storekeeper ☐ Trader ☐ Importer ☐ Exporter ☐ Packer:	
8. Coming from (city / State):	
9. Destination:	
10. Transportation:	*11. Crop:*
12. Lot size (kg or metric ton.):	
13. Lot nº:	*14. Date of collection:*
15. Sample size received by Laboratory (kg):	
16. Storage place:	
17. Storage condition:	
18. Sampling protocol number:	*19. Sample nº:*
20. Seal nº:	*21. Product registration nº:*
22. Sampling place (City / State):	
23. Analysis(es) requested:	
24. Nature of operation (objective of analysis): inspection / survey / other:	

25. Date of receipt: Day/Mo/Year	*26. Date of analysis: Day/Mo/Year*	*27. Date of issue: Day/Mo/Year*

Information supplied by customer, except fields no. 15, 25, 26 and 27.

Results of analysis

Mycotoxins analysed	*Results (µg/kg)*	*Method adopted*
		*
		*
		*
		*

**reference, Nd: not detected (LD < µg/kg). Maximum limit of X µg/kg for ochratoxin A, according to (reference). Statement on the estimated uncertainty of measurement, the recovery value and whether the result was corrected by the recovery.*

***The result(s) of this analysis is (are) restricted and apply only to sample(s) sent to laboratory by the customer, who is responsible (or not) for the sampling. The** Laboratory does (not) perform sampling.*

Partly reproduction of this document <u>is not authorised.</u>

Technician in charge of analysis
(name, function and signature)

Technician in charge of Laboratory
(name, function and signature)

Name and Address of laboratory where the tests were carried out Page x of y

Figure 34: Model of Analysis Report

II.2.10 REFERENCES

ANKLAM, E., STROKA, J., BOENKE, A. (2002) Acceptance of analytical methods for implementation of EU legislation with a focus on mycotoxins. *Food Control*, **13**, 173-183.

AOAC – Association of Official Analytical Chemist (2000) Natural Toxins. *Official Methods of AOAC International*. 2, 49, 1-64 and Appendix A. 17th edition. Edited by William Horwitz.

BOENKE, A. (1998) Method validation for mycotoxin determinations in food and foodstuffs. *Trendy in Analytical Chemistry*, **17**, 10-17.

CODEX Alimentarius Commission (2002) Codex committee on methods of analysis and sampling. Single-laboratory validation. Consideration of harmonized IUPAC Guidelines for the in-house validation of methods of analysis. CX/MAS 02/10.

CODEX Alimentarius Commission (2003) Codex committee on methods of analysis and sampling. ALINORM 03/23: Proposed draft guidelines on measurement uncertainty, Appendix V and Proposed draft guidelines for evaluating acceptable methods of analysis, Appendix VII.

EURACHEM (1998) The fitness for purpose of analytical methods – a laboratory guide to method validation and related topics. LGC (Teddington) Ltd. 61p.

EURACHEM (2000a) Selection, use and interpretation of proficiency testing (PT) schemes by laboratories. Produced by the EURACHEM Nederland and Laboratory of the Government Chemist (LGC), United Kingdom. 47p.

EURACHEM/CITAC (2000b) Guide: Quantifying Uncertainty in Analytical Measurement, Second edition. Editors: S L R Ellison, M Rosslein, A Williams. 120p.

EURACHEM/CITAC (2002) Guide to Quality in Analytical chemistry – an aid to accreditation. Prepared jointly by CITAC (The Cooperation on International Traceability in Analytical Chemistry) and EURACHEM (A Focus for Analytical Chemistry in Europe). 57p.

EURACHEM/CITAC (2003) Guide: Traceability in chemical measurement – a guide to achieving comparable results in chemical measurement. Editors: S L R Ellison, b King, M Rosslein, M Salit, A Williams. 37p.

CEN - European Committee for Standardization (1999) C*EN Report:* **CR 13505**:1999 E. 8p. Brussels.

FAPAS (2002) Food Analysis Performance Assessment Scheme – Report No. 1716, Ochratoxin Analysis, series 17, round 16, March 2002, UK.

FSA - Food Standards Agency (2004) Report to the standing committee on the food chain and animal health on the relationship between analytical results, the measurement uncertainty, recovery factors and the provisions in EU food and feed legislation with particular focus on the community legislation. SANCO/0064/2003-rev.3. *Information Bulletin on Methods of Analysis and Sampling for Foodstuffs*, nº 42, Norwich, United Kingdom.

GARFIELD, F. M., KLESTA E., HIRSCH J. (2000) Quality assurance principles for analytical laboratories, 3rd edition, *AOAC International*, Gaithersburg, MD USA. 187p.

GILBERT, J., ANKLAM, E. (2002) Validation of analytical methods for determining mycotoxins in foodstuffs, *Trends in analytical chemistry*, **21**, n. 67, 468-486.

GILBERT, J. (2002) Quality Control Measures for Mycotoxin Laboratories, *FDA Workshop on*

Mycotoxins, 22-26 July, 2002, Maryland, USA.

HORWITZ, W. (1988) Protocol for the design, conduct and interpretation of collaborative studies, *International Union of Pure and Applied Chemistry - IUPAC*, **60**, n. 6, 855-864.

ISO/AOAC/IUPAC (1999) Harmonised Guidelines for the Use of Recovery Information in Analytical Measurement. *Edited* Michael Thompson, Steven L R Ellison, Ales Fajgelj, Paul Willets and Roger Wood, *Pure Appl. Chem.*, **71**, 337-348.

ISO/AOAC/IUPAC (1995) International Harmonised Guidelines for Internal Quality Control in Analytical Chemistry Laboratories, Edited by M Thompson and R Wood, *Pure Appl. Chem.*, **67**, 649-666.

ISO GUM (1995) Guide to the Expression of Uncertainty in Measurement (revised edition) International Organisation for Standardization, Geneva.

ISO 5725 (1994) *Accuracy (trueness and precision) of measurement methods and results*, parts 1-6, International Organisation for Standardization, Geneva.

ISO Guide 43-1 (1997) Proficiency testing by intercomparisons – Parts 1 and 2, International Organisation for Standardization, Geneva.

ISO 8402 (1994) *Quality - Vocabulary*, International Organization for Standardization, Geneva.

ISO/IEC 17025 (1999) *General requirements for the competence of calibration and testing laboratories*, International Organisation for Standardisation, Geneva.

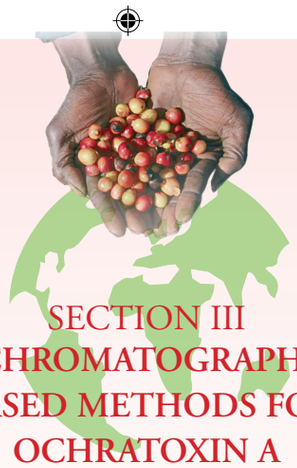
IUPAC – International Union of Pure and Applied Chemistry (1995) IUPAC Harmonized Protocols for the adoption of standardised analytical methods and for the presentation of their performance characteristics. *Pure and Applied Chemistry*, **62**, 149-162.

MAPA (2001a) Ministério da Agricultura, Pecuária e Abastecimento. LACQSA/LAV-MG - Laboratório de Controle de Qualidade e Segurança Alimentar *Quality Manual*, edition 02, revision 01, and related documentation, Brazil. 700 p.

MAPA (2001b) Ministério da Agricultura, Pecuária e Abastecimento. LACQSA/LAV-MG - Laboratório de Controle de Qualidade e Segurança Alimentar. Standard Operational Procedure (*SOP 039 ed. 02, rev. 01*): *Determination of ochratoxin A In green coffee by immunoaffinity column clean up with LC and TLC*, Brazil. 13 p.

FAO - Material for the Training Course "Development of quality assurance for mycotoxin analysis of food and feed", Food and Agriculture Organization of the United Nations and International Atomic Energy Agency, Instituto Adolfo Lutz, São Paulo, May 13-19/2000, prepared by Dr. Maya Piñeiro.

THOMPSON, M, WOOD, R. (1993) International Protocol for Proficiency Testing of (Chemical) Analytical Laboratories. *Journal of AOAC International*, **76**, 4, 926-940.



SECTION III
CHROMATOGRAPHY BASED METHODS FOR OCHRATOXIN A

III.1. INTRODUCTION

Ochratoxin A {R-N-[5-chloro-3,4-dihydro-8-hydroxi-3-methyl-1-oxo-1H-2-benzopyran-7-yl)carbonyl]-l-phenylalanine}(Figure 1) has been classified as a substance of the group 2B by IARC (1993) meaning the existence of sufficient evidence of its renal carcinogenicity to animals and possibly to humans. The Joint Expert Committee on Food Additives (JECFA) established the Provisional Tolerable Weekly Intake (PTWI) of 100 ng/kg body weight based on the lowest amount of the toxin (0.008 mg/kg bw per day and a safety factor of 500) that causes adverse effects to swine kidney (WHO 2001).

Cereals and cereal products have been reported as the major contributors for the intake of ochratoxin A. Other sources of ochratoxin A in the diet have been reported as being wine, green and roasted coffee, grape juice, cocoa and chocolate, oils, olive, pulses, meat products, nuts, dried fruits, beer, among others. The toxin is mainly produced by *Penicillium verrucosum,* by *Aspergillus ochraceus (*the main source of ochratoxin A in green coffee), and several related *Aspergillus* species, and by *A. carbonarius* (WHO 2001).

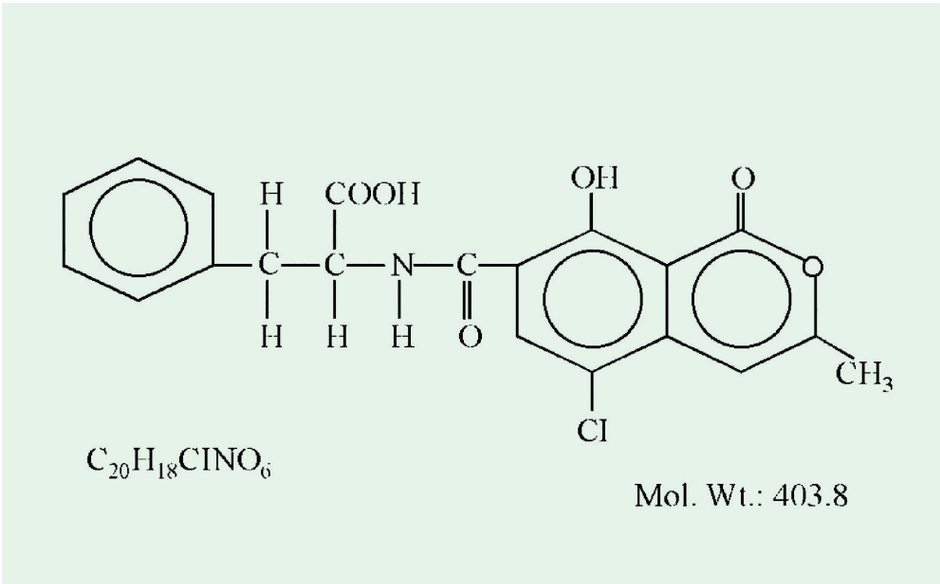


Figure 1: Chemical structure of ochratoxin A

Differently from aflatoxins, the analysis of ochratoxin A by TLC was restricted to surveys in the 1970´s due to lack of precision and sensitivity of the method. From 1980´s onwards, liquid chromatography was the method of choice for assessing ochratoxin A in coffee (Table 1). The proposed European Union regulation for the presence of ochratoxin A in green and roasted coffee, along with sampling plans and method performance criteria [as a modification of EC directives no. 2002/472/CE (CEE 2002a) and 2002/26/CE (CEE 2002b), respectively], has recently provided the drive to improve analytical methods and extend validation. Two methods for detection of ochratoxin A in green coffee and roasted coffee were recently internationally validated (Entwisle *et al*. 2001, Vargas *et al*. 2002).

In this very short review we have tried to give an overview of the analytical methods and have covered sampling, which cannot be separated from the analytical determination. We have compiled information on chromatographic methods for determining ochratoxin A and have assessed not only performance from the stand-point of formal validation but also included information, where available, on in-house validation.

III.2. SAMPLING PLAN

There is general recognition of the importance of sampling and that meaningful results can only be obtained if representative samples are taken and properly homogenised prior to sub-sampling for analysis. Despite of this recognition, sampling is still much neglected, and often in the drive for rapid methods, because sampling and sample preparation is very time-consuming, proper sampling is frequently overlooked. In order to have meaningful results, representative samples need to be collected by using a clearly defined sampling plan (Gilbert and Vargas 2003). Although the description of a number of sampling plans for aflatoxins, deoxynivalenol, fumonisins in different products have been published only recently a sampling plan for determining ochratoxin A in green coffee was designed. In this sampling plan, variances associated with testing of a lot of green coffee for OTA using a 1 kg sample, Romer RAS type mill, 25 g sub-sample, and HPLC analytical method was estimated. Sampling, sample preparation and analytical variances were 7.80, 2.84, 0.11, respectively and account for about 73%, 26%, and only 1% of the total variability, respectively, which is consistent with what has been observed with other mycotoxins and other commodities such as corn and peanuts (Vargas *et al*. 2003). The 2-parameter lognormal function was established for the distribution of ochratoxin A in green coffee and the operational characteristic curves calculated and exporter's risk or false positives and importer's risk or false negatives were estimated. The EU proposed regulatory limit of 5 and 10 μg/kg for ochratoxin A in green coffee was used as the accept/reject limit i.e. the threshold concentration that separated good lots from bad lots (data not published).

III.3. EXTRACTION AND SAMPLE CLEAN UP

The major problems associated with most analytical methods for determination of ochratoxin A is the extraction of co-extractives with potential to interfere in the analysis, which requires an efficient clean-up step before quantification.

A variety of solvents and clean-up procedures have been used in the latest 30 years attempting to assess ocratoxin A contamination in green, roasted and/or soluble coffee, mainly by LC (Table 1).

Attempts have been made to enhance the sensitivity, selectivity and safety of ochratoxin A. Substitution of the highly toxic benzene with toluene for preparation of ochratoxin A standard solutions has been accomplished with calculation of molar absorptivities in toluene-acetic acid (9+1, v/v) (Trucksess 1999). Toxic chlorinated solvents (Levi *et al*. 1974, Levi 1975, Patel *et al.* 1997) have been replaced by alternative extractants. Ochratoxin A is nowadays usually extracted with organic solvent and water or a mixture of both, containing small amount of acid (Studer-Rohr *et al.* 1995, Pittet *et al.* 1996, van der Stegen *et al.* 1997, Trucksess *et al.* 1999). The combination of aqueous methanol and bicarbonate has been the preferred extraction solvent (Pittet *et al.* 1996, Entwisle *et al.* 2001). However, notwithstanding these changes, the long-established AOAC 975.38 method and its extraction step variation are still employed and continue to be recommended by AOAC International (2000).

At the turn of the millennium, conventional clean up procedures such as liquid-liquid partition (Pittet and Royer 2002) are still employed along or in combination with solid–phase extraction, in particular, in combination with the laborious and time-consuming celite column chromatography (Levi *et al.* 1974, Levi 1975, Cantáfora *et al.* 1983, Micco *et al.* 1989, Studer-Rohr *et al.* 1994, 1995).

Other conventional solid phase materials such as the surface-modified bonded silica like C_{18}, (Terada *et al.* 1986), aminopropyl, trimethy aminopropyl, n-propyl-ethylene-diamine, cyanopropyl and diol (Sibanda *et al*. 2002b), DEA - anion exchange column (Akyama *et al.* 1997) have been introduced as a clean-up step for ochratoxin A analysis. Among the solid-phases (aminopropyl, trimethy aminopropyl, n-propyl-ethylene-diamine, cyanopropyl and diol) studied by Sibanda *et al*. (2002b) only aminopropyl was efficient to remove the brown interferences from roasted coffee. Neither false positive nor false negative was determined in the analysis of ochratoxin A by HPLC and flow-through enzyme immunoassay when aminopropyl was used as a clean up step. The method is recommended to screen roasted coffee samples using a cut off point of 4 μg/kg.

Great improvements have been achieved in ochratoxin A analysis with the use of immunoaffinity solid-phase extraction (SPE) sorbents (Pittet *et al.* 1996, Nakajima *et al.* 1997, Nakajima 2003). The immunosorbents, through improved selectivity in the SPE step have allowed the development of highly selective methods with detection limits as low as 0.1-0.2 ng/g (Pittet *et al.* 1996, Vargas *et al.* 2002). Immunoaffinity column clean up has been shown to be a robust technique for purification, separation and concentration of ochratoxin A in green, roasted and soluble coffee (Pittet *et al.* 1996) with mean recoveries of 99, 93, 92%, respectively and a coefficient of variation (CV) varying from 3.5 to 14.3%. From 1996 onwards, Pittet´s method has been extensively employed by control laboratories and industries all over the world for assessing ochratoxin A in green coffee as given in Table 1.

van der Stegen *et al.* (1997) have reported that in the screening exercises for assessing ochratoxin A in European final products the 08 European laboratories had used HPLC with fluorescence detector as a quantification step. The biggest analytical differences lied on the extraction and clean

up step. Some laboratories used a sole immunoafinity column; or either a combination of liquid-liquid partition and celite column chromatography or a combination of liquid-liquid partition with immunoaffinity columns. A CV of 42% was determined among the laboratories for the analysis of a coffee test sample at ochratoxin A mean level of 4.2 μg/kg, which was higher than the value expected by Horwitz equation (36%). With the formal validation of analytical method for roasted coffee using a combination of phenyl silane and immunoaffinity (Entwisle *et al*. 2001) it was shown that the variability of test results among the laboratories was 20-29% (RSD_R).

The disadvantage associated with the newest solid-phase immunoaffinity columns is the cost. However, there is a possibility for re-use of immunoaffinity columns (Nakajima *et al.* 1997, Leoni *et al*. 2000, Santos and Vargas 2002). Immunoaffinity column clean up is quite straightforward, can be easily automated, making possible a high throughput of samples per run of analysis. The time saved during daily routine analysis counts as a distinct advantage over a number of commercially available SPE columns.

III.4. SEPARATION AND QUANTIFICATION STEPS

Liquid chromatography (LC), including high performance liquid chromatography (HPLC) and thin layer chromatography (TLC) have been so far the most widely accepted techniques for separation of ochratoxin A from many types of foods (Lin *et al.* 1998).

TLC has been the most widely used and established separation and detection technique for aflatoxins since its development in 1960s. However, the same does not apply for ochratoxin A analysis. The AOAC official method date back from 1975 and was extensively used until the 1980´s when LC was introduced. Some of the factors affecting the acceptance of TLC as a quantitative method such as its lack of resolution and poor sensitivity (AOAC 2000, Pittet and Royer 2002) have been overcome in recent years.

The combination of a robust immunoaffinity column clean-up with the advantageous low cost TLC for analysis of ochratoxin A has shown to be a promising analytical approach and has dramatically changed the analytical perspective for ochratoxin A determination by providing sample extracts free of major matrix interferences and suitable for one dimensional TLC analysis, making the TLC method more straightforward and amenable to automation. Detection limits as low as 0.5 μg/kg for ochratoxin A, and method performance characteristics meeting standards required by international organisations (CEN 1999) have been achieved (Santos and Vargas. 2002) (Table 1).

TLC should always be considered an important tool as it is fast, cost effective and can be used in routine applications (crude extract analysis, versatility in using different solvent systems, applicable to different visualisation system using the same sample extract. TLC allows those in developing countries to assess ochratoxin A contamination irrespective of the purpose of the assessment whether qualitative (Pittet and Royer 2002) or quantitative (Santos and Vargas 2002). New attempts in the development and validation of new methods based on the solid-phase extraction and TLC should be achieved in order to improve the analytical capabilities in developing countries.

However, quantification is still a limiting factor due to the high cost of commercial fluorodensitometers, which could otherwise decrease the variability associated with the ability of individual analysts to visually quantify the toxin (Castro and Vargas 2001, Pittet and Royer 2002, Santos and Vargas 2002). Successful attempts have been made (Stroka *et al*. 2000) to develop alternative devices in regard of the expensive commercial TLC densitometers for aflatoxin analysis that could be validated for ochratoxin A. These developments could be extremely helpful in building the ability to analyse ochratoxin A in developing countries, especially if the densitometers could be available on a semi-commercial scale.

Although the analysis of ochratoxin A in coffee has had a substantial improvement with the introduction of LC as a modification of the AOAC official method quantification step (Cantáfora *et al*. 1983) by reducing the detection limit of the methods by 10-20 times, the great improvement in the analysis of ochratoxin A by LC was the combination of immunoaffinty (IAC) as clean-up step (Nakajima *et al.* 1990) with reversed-phase HPLC. It has been since then the most attractive approach to assess ochratoxin A contamination in coffee (Pittet *et al.* 1996, Nakajima *et al.* 1997, Patel *et al.* 1997, Scott and Trucksess 1997, Jørgensen 1998, Trucksess *et al.* 1999), giving clean extracts, well defined chromatograms with no interference at ochratoxin A retention time. Nevertheless, interferences such as caffeine and/or a ochratoxin A diastereomer are still reported in the analysis of roasted coffee by IAC with HPLC and the use of phenyl silane and aminopropyl as solid phase in combination with IAC has been proposed (Entwisle *et al.* 2001, Sibanda *et al.* 2002a). Lombaert *et al*. (2002) indicate that some interferences counts to higher incidence of ochratoxin A in samples analysed by IAC when compared to phenyl silane/IAC. However, the recoveries for spiked samples for both clean up steps were equivalent. Santos and Vargas (2002) did not report any difference in the ochratoxin A content in green coffee samples fortified with three levels of caffeine and cleaned up on IAC.

Acidic mobile phases have been preferable for separation of ochratoxin A by LC. Because ochratoxin A is a polar compound, which has a carboxyl group in the structure, it must be chromatographed in an ionised form. Fluorescence intensity increases with the increasing pH value, but ochratoxin A is not retained under neutral and alkaline conditions from reversed phase chromatography that usually can not stand pH higher than 8 (Terada *et al*. 1986). The use of an ion-pair chromatography enables the high polar compounds to act as weak polar compounds. Capcell Pak C_{18} (silicone coated C_{18} column) has been reported to stand pH as high as 10, allowing the increase the sensitivity 80-fold (Nakajima *et al*. 1990).

The main advantage of HPLC lies in its possibility of automation, separation power, selectivity and low detection limit achievement, although HPLC is expensive and requires skilled and experienced staff to operate and maintain the equipment. Detection limits of 20, 10 and 0.5 µg/kg were reported for TLC methods by Levi *et al.* (1974), Pittet and Royer (2002) and Santos and Vargas (2002), respectively, whilst lower limits of detection of 0.12-0.2 µg/kg (Pittet *et al.* 1996, Vargas *et al.* 2002) and 0.5 - 2 µg/kg (Terada *et al.* 1986, Tsubouchi *et al.* 1988, Studer-Rohr *et al.* 1994, 1995) have been achieved by employing HPLC technique.

III.5. CONFIRMATION OF OCHRATOXIN A

Confirmation of ochratoxin A by TLC has been achieved by spraying the TLC plates with aluminium chloride and sodium bicarbonate or by exposing the plates to ammonia vapour (Levi 1975, AOAC 2000, Santos and Vargas 2002). RP TLC has been reported as a confirmation method for ochratoxin A in coffee appearing in normal-phase TLC (Santos and Vargas 2002) or as preparative chromatography for HPLC (Frohlich *et al.* 1988) for matrices other than coffee.

Confirmation of ochratoxin A by HPLC has been carried out mostly by ochratoxin A methyl ester formation using boron trifluoride (Cantáfora *et al.* 1983, Pittet *et al.* 1996) or sulphuric (Terada *et al.* 1986, Tsubouchi *et al.* 1988) and chloride acids (Studer-Rohr *et al.* 1994) and diazomethane (Studer-Rohr *et al.* 1995). The use of sep-pak NH_2 cartridge with LC as a confirmatory procedure for contaminated samples already cleaned up by liquid partition in combination with C_{18} sep-pak has been reported (Tsubouchi *et al.* 1988).

A novel procedure GC - negative ion chemical ionisation (NICI) MS and multiple ion detection (MDI) modes using the hexadeutered O-methyl-d_3-ochratoxin A methyl-d_3 ester derivative, as internal standard for confirmation of ochratoxin A in contaminated food by converting into its O-methylocratoxin A methyl ester derivative (OA-Me_2) at level of 0.1 μg/kg has been demonsrated (Jiao *et al.* 1992). Studer-Rohr *et al.* (1995) has reported the combination of diazomethane methylation with GC/MS (CCI/MID) for the confirmation of ochratoxin A identity in roasted coffee.

Liquid chromatography tandem mass spectrometry (LC-ESI-MS-MS) in combination with SRM has been employed as confirmation procedure for ochratoxin A in coffee (Becker *et al.* 1998). Lombaert *et al.* (2002) used LC-MS-MS for confirmation of ochratoxin A with good agreement with HPLC results. According to Ventura *et al.* (2003) the use of triple-quadrupole detector MS is not necessary as good agreement between HPLC and HPLC-ESI-MS is achievsble.

Tuomi *et al.* (2001) have reported a method for simultaneous detection of several mycotoxins including ochratoxin A in a building material matrix using HPLC with tanden mass spectrometric identification and quantification using ESI-MS-MS.

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III.6. VALIDATION OF ANALYTICAL METHODS FOR OCHRATOXIN A

Despite of the number of papers published on ochratoxin A work in coffee from 1974 to 2003 (Table 1), very few ones were carried out with either formally validated or in-house validated method. Usually, only recovery data using a spiked sample were reported and no data concerning method performance were possible to assess from a large number of papers. In fact, the AOAC 975.38 validated method dates back from 1975 (AOAC 2000), and only recently two methods for both roasted (Enstwisle *et al.* 2001) and green coffee (Vargas *et al.* 2002) have been thoroughly internationally collaboratively validated using the International Harmonised Protocol (Thompson and Wood 1993, IUPAC 1995) and comply with future EU regulation for ochratoxin A in green coffee in terms of applicability range, recovery rate and RSD_r , RSD_R, Horrat values.

Table 1. Methods for determination of ochratoxin A in coffee

					Method performance				
Year	Matrix	Cleanup	Detection/ Quantification	Confirmation	LD/LQ (µg/kg)	Recovery (%)	Relative standard deviation (%)	Range of contamination (µg/kg)	Reference
1974	Green coffee	CC:celite/ bicarbonate	Visual TLC	NH_3, $AlCl_3$, $NaHCO_3$,	Not given	83.5 (55-101)	Not given	23-221	Levi *et al* 1974
1975	Green coffee	CC:celite/ bicarbonate	Visual TLC	NH_3, $AlCl_3$, $NaHCO_3$,	20/	69 (60.5-84.6)	RSR_r: 21-32 RSD_R:16-25	57-230 (n=3)	Levi 1975 Collaborative Study
1976	Inoculated Green coffee	Liquid partition	Visual TLC	Not given	Not given	Not given	Not given	Not given	Gallaz and Stalder 1976
1980	Green coffee	CC:celite/bicarbonate	Visual TLC	NH_3, $AlCl_3$, $NaHCO_3$,	20/	Not given	Not given	Not given	AOAC 975.38 AOAC 2000
1983	Inoculated Green coffee	Liquid partition	TLC Densitometer	Not given	Not given	Not given	Not given	Not given	Tsubouchi *et al.* 1983
1983	Green	Liquid partition CC:celite/bicarbonate	HPLC	Esterification Alcohol/ BF_3	0.2/0.4	69.5-82.3	5.6-7.1	5-10	Cantáfora *et al.* 1983
1986	Instant Green Roasted Drink	Liquid partition/ SPE: C18 sep-pak	HPLC	Esterification Alcohol/H_2S0_4	5/ 2/ 2/ 0.2/	80.7-89.5 81.5 89.7 92.1	3.74-4.26 5.93 3.43 5.78	100, 10 20 20 2	Terada *et al.* 1986
1987	Green Coffee brew	Liquid partition CC:celite/bicarbonate	TLC Densitometer	Not given	Not given	Not given	Not given	Not given	Tsubouchi *et al.* 1987
1988	Roast Ground	Liquid partition/ SPE: C18 sep-pak	HPLC	NH_3Sep-pak Esterification Alcohol/H_2S0_4	Not given	Not given	Not given	Not given	Tsubouchi *et al.* 1988

Table 1. Methods for determination of ochratoxin A in coffee

					Method performance				
Year	Matrix	Cleanup	Detection/ Quantification	Confirmation	LD/LQ (µg/kg)	Recovery (%)	Relative standard deviation (%)	Range of contamination (µg/kg)	Reference
1989	Green Roasted	Liquid partition CC:celite/ bicarbonate	HPLC	Esterification BF_3	0.01/	90-95 Not given	2.4 Not given	5 -10 Not given	Micco *et al.* 1989
1990	Green Roast Instant Canned drink	IAC in-house prepared	HPLC	C18 sep-pak and Esterification Alcohol/H_2S0_4	0.5/ - 0.5/ 0.025/	102.6 99.4 102.8 104.1	1.11 4.56 4.42 3.49	20 (n=5) 20 (n=5) 20 (n=5) 1 (n=5)	Nakajima *et al.* 1990
1994	Green Roasted Coffee brew	Liquid partition CC:celite/ bicarbonate	HPLC	Esterification Alcohol/HCl and additional HPLC GC/MS IAC and GC/MS Methyl ester	0.5/ 1.0/ 1.0/	Not given	Not given	Not given	Studer-Rohr *et al.* 1994
1995	Green Roasted Coffee brew	Liquid partition CC:celite/ bicarbonate	HPLC	Esterification Alcohol/HCl IAC and additional HPLC/Methyl ester GC/MS Methyl ester-diazomethane	0.5/ 1.0/ 1.0/	97 116 87	5-12	Not given (n=3)	Studer-Rohr *et al.* 1995
1996	Green	IAC and C_{18}	HPLC	Not given	/0.26	73.3	18	Not given	MAFF 1996
1996	Green Roasted Soluble	IAC	HPLC	Methyl ester BF_3	0.2/	99 (90-108) 93 (89-100) 92 (80-103)	0.5-5.0	4.8-13.0 (n=5) 25.7-14.3 (n=5) 3.5-12.4 (n=5)	Pittet *et al.* 1996

Table 1. Methods for determination of ochratoxin A in coffee

					Method performance				
Year	Matrix	Cleanup	Detection/ Quantification	Confirmation	LD/LQ (µg/kg)	Recovery (%)	Relative standard deviation (%)	Range of contamination (µg/kg)	Reference
1997	Instant Roasted Coffee Brew	Liquid partition CC:celite/ bicarbonate with and without IAC Only IAC Silica sep-pak	HPLC	Not given	0.2-1.0/ (9 laboratories)	Not given	NC (naturally contaminated sample)	42 20 93 (overall CV: 08 laboratories	van der Stegen *et al.* 1997
1997	Green	IAC (0.8-1.0 µg binding capacity)	HPLC	Not given	0.1/	98	Not given	10	Nakajima *et al.* 1997
1997	Green	DEA (anion exchange column)	HPLC	Methyl ester BF_3	-	-	-	-	Akyama *et al.*1997
1997	Soluble and Roasted	CC: silica gel and IAC Silica sep-pak (modified CC method) and IAC	HPLC	Methyl ester Boron trifluoride	0.1/	91 (70-110) 84 (70-98) 87	5.1 4.8	2 (n=10) 2 (n=41) (matrix Not given) 2 (n=10)	Patel *et al.* 1997
1998	Green	IAC	HPLC	Methyl ester BF_3	Not given	Not given	Not given	Not given	Bucheli *et al.* 1998
1998	Not specified	Silica sep-pak cartridge	LC-ESI-MS-MS	-	-	70	12.8	Not given	Becker *et al.* 1998
1998	Green Roast Soluble	IAC	HPLC	Methyl ester BF_3	0.2/	Not given	Not given	Not given	Blanc *et al.* 1998
1998	Roasted	IAC	HPLC	Postcolumn 6%amonia	0.1/	75 (59-83)	Not given	5 (n=6)	Jørgensen 1998
1999	Green Roasted	IAC	HPLC	Methyl ester Boron trifluoride GC/MS	0.03/	86-90 75-81	2.3-8.8 3.1-3.8	1 - 4 (n=3)	Trucksess *et al.* 1999
2000	Green	Liquid partition/ SPE: C18 sep-pak	HPLC	Not given	0.1/	89	5.6 4.9-17.1(NC)	10 (n=4)	Romani *et al.* 2000
2000	Green	IAC	HPLC	Methyl ester BF_3	Not given	Not given	Not given	Not given	Bucheli *et al.* 2000

Table 1. Methods for determination of ochratoxin A in coffee

					Method performance				
Year	Matrix	Cleanup	Detection/ Quantification	Confirmation	LD/LQ (µg/kg)	Recovery (%)	Relative standard deviation (%)	Range of contamination (µg/kg)	Reference
2000	Roasted Instant	IAC	HPLC	Not given	0.25/0.80	80 (73-86) 82 (76.5-87)	8.5-14 7.23-7.75	4.2-8.4 1.5-10	Prado *et al.* 2000
2000	Roasted Instant	IAC	HPLC	Not given	0.2/	98-100	Not given	10	Leoni *et al.* 2000
2001	Green and roasted	IAC	HPLC	Methyl ester BF_3	0.5/	84	11 (CV)	3.1 2.4 (NC)	Van der Stegen *et al.* 2001
2001	Roasted	Phenyl silane /IAC	HPLC	Not given	0.1/	85 (65-97)	RSR_r: 6 RSD_R:13 RSR_r: 2-27 RSD_R:14-71	3.5 0.1-5.4	Entwisle *et al.* 2001 AOAC Collaborative Study
2002	Roasted	Aminopropyl (NH_2)/IAC	HPLC	Methyl ester Boron trifluoride	Not given	72-84	Not given	10 (n=3)	Sibanda *et al.* 2002a
2002	Roasted	Aminopropyl (NH_2)/IAC	HPLC Flow-through enzyme immunoassay	Not given	Not given	73-87	Not given	10 (n=3)	Sibanda *et al.* 2002b
2002	Green	Liqui-liquid partition	TLC visual Two dimensional	Rf and spot fluorescence	10/	Qualitative Not given Good agreement with HPLC results	Not given	5,10,20 (n=2)	Pittet and Royer 2002
2002	Green	IAC	TLC visual	Sodium bicarbonate	0.5/	82-109	0.0-18.8 0.0-14.4	1.8 -109 (n=3) (spiked) 4.1,9.5,12.5 (n=3) NC	Santos and Vargas 2002

Table 1. Chromatographic Methods for determination of ochratoxin A in coffee

					Method performance				
Year	Matrix	Cleanup	Detection/ Quantification	Confirmation	LD/LQ (µg/kg)	Recovery (%)	Relative standard deviation (%)	Range of contamination (µg/kg)	Reference
2002	Green	IAC	TLC densitometry	Sodium bicarbonate	0.5/	83.7-133	– 24.9 9.3-20.3	1.8 -109 (n=3) (spiked) 4.1,9.5,12.5 (n=3) naturally contaminated	Santos and Vargas 2002
2002	Green	IAC	HPLC	Not given	0.1/	85 (65-97)	RSR_r: 7.42 RSD_R:16.34 RSR_r: 9-16 RSD_R:20.-29	4.48 2.60, 6.32, 12.89	Vargas *et al.* 2002 Collaborative Study
2002	Roasted ground Instant	IAC Phenyl silane/IAC	HPLC	Methyl ester LC-tandem mass soectrometry (MS/MS)	0.1/	70-89	60-116 64-111	2 (n=23)	Lombaert *et al.* 2002
2003	Green	IAC	HPLC	Not given	0.2/	Not given	Not given	Not given	Taniwaki *et al.* 2003
2003	Green	Phenyl silane /IAC	HPLC	Not given	0.2/	92-98.7	Not given	1,2,4,10 (n=2)	Martins *et al.* 2003
2003	Green and roasted	Oasis MAX Polymeric column	HPLC	HPLC/ESI	0.1/0.5	82.4	4 (CV) 4.9 (intermediate precision)	3	Ventura *et al.* 2003
2003	Green and roasted	IAC	HPLC	Not given	0.1/	96.8 109.5	4.9 2.1	20 (n=4)	Romani *et al.* 2003

TLC: Thin Layer Chromatography; HPLC: High Performance Liquid Chromatography; MS: Mass spectrometry; SPE: Solid Phase Chromatography; IAC: Immunoaffinity Column; CC: Column Clean-up

III.7. REFERENCES

AKYAMA, H. CHEN, D., MIYAHARA, M., GODA, Y., TOYODA, M. (1997) A rapid analysis of ochratoxin A in coffee beans and cereals. The Journal of Food Hygienic Society of Japan, **38** (6), 406-411.

AOAC – Association of Official Analytical Chemist (2000) Natural Toxins. Official Methods of AOAC International. Volume 2, Chapter 49, 1-64. 17th edition. Edited by William Horwitz.

BECKER, M., DEGELMANN, P., HERDERICH, M., HUMPI, H-U. (1998) Column chromatography – electrospray ionisation – tandem mass spectrometry for the analysis of ochratoxin. Journal of Chromatography A, **818**, 260-264.

BLANC, M., PITTET, A., MUNOZ-BOX, R., VIANI, R. (1998) Behaviour of ochratoxin A during green coffee roasting and soluble coffee manufacture. J, Agric. Food Chem. **46,** 673-675.

BUCHELI, P., KANCHANOMAI, C., MEYER, I., PITTET, A. (2000) development of ochratoxin a during Robusta (Coffea canephora) coffee cherry drying. J, Agric. Food Chem. **48,** 1358-1362.

BUCHELI, P., MEYER, I., PITTET, A., VUATAZ, G., VIANI, R. (1998) Industrial storage of green robusta coffee under tropical conditions and its impact on raw material quality and ochratoxin A content. J, Agric. Food Chem. **46,** 4507-4511.

CANTÁFORA, A., GROSSI, M., MIRAGLIA, M., BENELLI, L. (1983) Determination of ochratoxin A in coffee beans using reversed-phase high performance liquid chromatography. Riv. Soc. Ital. Sci. Aliment., **12**, 103-108.

CASTRO, L., VARGAS, E. A. (2001) Determining aflatoxins B_1, B_2, G_1, G_2 in maize using florisil clean up with thin layer chromatography and visual and densitometric quantification. Food Science and Technology **21,** 115.

CEE - European Commission. (2002a) Regulamento (CE) No. 472/2002 da Comissão de 12 de março de 2002. Jornal Oficial das Comunidades Européias. L75/18-20.

CEE - European Commission. (2002b) Directiva 2002/26/CE da Comissão de 13 de março de 2002. Jornal Oficial das Comunidades Européias. L75/38-43.

CEN – European Committee for Standardization (1999) CEN Report: Food Analysis. Biotoxins: Criteria of analytical methods of mycotoxins, Brussels. CR 13505:1999 E. 8p.

ENTWISLE, A. C., WILLIAMS, A. C., MANN, P. J., RUSSEL, J., SLACK, P. T., GILBERT, J. (2001) Combined phenyl silane and immunoaffinity column clean-up HPLC for the determination of ochratoxin A in roasted coffee: Collaborative study. Journal of AOAC International, **84**, 444-450.

FROHLICH, A. A., MARQUARDT, R. R., BERNATSKY, A. (1988) Quantification of ochratoxin A: Use of reverse phase thin-layer chromatography for sample clean-up followed by liquid chromatography or direct fluorescence measurement. Journal of Association of Official Analytical Chemist, **71**, 949-953.

GALLAZ, L., STALDER, R. (1976) Ochratoxin A im kaffee. Chem. Mikrobiol. Technol. Lebensm. **14**, 147-149.

GILBERT, J. VARGAS, E.A. (2003) Advances in Sampling and analysis for aflatoxins. In: Journal of Toxicology - Toxins Reviews 2003, **22** (issues 2 & 3) pp 385-426. Marcel Dekker INC. DOI: 10.1081/TXR-120024099

IARC - International Agency for Research on Cancer (1993) *Ochratoxin A, IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*: *Some Naturally Occurring Substances, Food Items and Constituents, Heterocyclic Aromatics Amines and Mycotoxins* (Lyon: International Agency for Reseach on Cancer), pp.489-521.

IUPAC – International Union of Pure and applied Chemistry, 1995, Harmonized guidelines for internal quality control in analytical chemistry laboratory. Pure and Applied Chemistry, **67**, 649-666.

JIAO, Y., BLAAS, W., RUHL, C., WEBER, R. (1992) Identification of ochratoxin A in food samples by chemical derivatization and gas chromatography – mass spectrometry. Journal of Chromatography, **595**, 364-367.

JØRGENSEN, K. (1998) Survey of pork, poultry, coffee, beer and pulses for ochratoxin A. *Food Additives and Contaminants*, **15**, 550-554.

LEONI, L. A. B., SOARES, V., M., L., OLIVEIRA, P., L., C. (2000) Ochratoxin A in Brazilian roasted and instant coffee. Food Additives and Contaminants, **17**, 867-870.

LEVI, C. P., TRENK, H. L., and MOHR, H. K. (1974) Study of the occurrence of ochratoxin A in green coffee beans. Journal of Association of Official Analytical Chemist, **57**, 866-871.

LEVI, C.P. (1975) Collaborative study of a method for the determination of ochratoxin A in green coffee. Journal of Association of Official Analytical Chemist, **58**, 2, 258-262.

LIN, L, ZHANG, J., WANG, P., WANG, Y., JIPING, C. (1998) Thin-Layer Chromatography of mycotoxins and comparison with other chromatographic methods. *Journal of Chromatography A*, **815**, 3-20.

LOMBAERT, G. A., PELLAERS, P., CHETTIAR, M., LAVALEE, D., SCOTT, P.M, LAU, B.P.-Y. (2002) Survey of Canadian retail coffees for ichratoxin A. Food Additives and Contaminants, **19** (9) 869-877.

MAFF Food Safety Directorate (1996) Surveillance of ochratoxin A in green (unroasted) coffee beans. Food Surveillance Information Sheet, **80**, March.

MARTINS, M. L., MARTINS, H. M., GIMENO, A. (2003) Incidence of microflora and of ochratoxin A in green coffee beans (Coffea Arabica). Food Additives and Contaminants, **20** (12), 1127-1131.

MICCO, C., GROSSI, M., MIRAGLIA, M., BRERA, C. (1989) A study of the contamination by ochratoxin A of green coffee and roasted coffee beans. Food Additives and Contaminants, **6** (3), 333-339.

NAKAJIMA, M. (2003) Studies on mycotoxin analysis using immunoaffinity column. *Mycotoxins,* **53**, 43-51.

NAKAJIMA, M., TERADA, H., HISADA, K., TSUBOUCHI, H., YAMAMOTO, K., UDA, T., ITOH, Y., KAWAMURA, O., and UENO, Y. (1990) Determination of ochratoxin A in coffee beans and coffee products by monoclonal antibody affinity chromatography. Food and Agricultural Immunology, **2**, 189-195.

NAKAJIMA, M., TSUBOUCHI, H., MIYABE, M., UENO, Y. (1997) Survey of aflatoxin B_1 and ochratoxin A by high-performance liquid chromatography linked with immunoaffinity chromatography. Food and Agricultural Immunology, **9**, 77-83.

PATEL, S., HAZEL, C. M., WINTERTON, A. G. M., GLEADLE, A. E. (1997) Survey of ochratoxin

A in UK retails coffee. Food Additives and Contaminants, **14** (3), 217-222.

PITTET, A., ROYER, D. (2002) Rapid, low cost thin layer chromatographic screening for the detection of ochratoxin A in green coffee at control level of 10 µg/kg. *Journal of Agricultural and Food Chemistry*, **50**, 243-247.

PITTET, A., TORNARE, D., HUGGETT, A., VIANI, R. (1996) Liquid chromatographic determination of ochratoxin A in pure and adulterated soluble coffee using an immunoaffinity column clean up procedure. *Journal of Agricultural and Food Chemistry,* **44**, 3564-3569.

PRADO, G., OLIVEIRA, M. S., ABRANTES, F. M., SANTOS, L. G., VELOSO, T., BARROSO, R. E. S. (2000) Incidência de ocratoxina A em café torrado e moído e em café solúvel consumido em Belo Horizonte, MG. *Ciência e Tecnologia de Alimentos*, 20 (2), 192-196.

ROMANI, S., PINNAVAIA, G. G., ROSA, M. D. (2003) Influence of roasting levels on ochratoxin A content in coffee. *J. Agric. Food Chem*. **51**, 5168-5171.

ROMANI, S., SACHETI, G., LÓPEZ, C.C., PINNAVAIA, G. G., ROSA, M. D. (2000) Screening on the occurrence of ochratoxin A in green coffee beans of different origins and types. *J. Agric. Food Chem.* **48**, 3616-3619.

SANTOS, E. A., VARGAS, E. A., 2002, Immunoaffinity column clean-up and thin layer chromatography for the determination of ochratoxin A in green coffee. *Food Additives and Contaminants*, **19**, 447-458.

SCOTT, P. and TRUCKSESS, M. W. (1997) Application of immunoaffinity columns to mycotoxin analysis. *Journal of AOAC International*, **80**, 941-949.

SIBANDA, L., De DAEGER, S., BARNA-VETRO, I., Van PETEGHEM, C. (2002b) Optimization of solid-phase clean-up prior to liquid chromatographic analysis of ochratoxin A in roasted coffee. *Journal of Agric Food Chem*, **50**, 6964-6967.

SIBANDA, L., De DAEGER, S., Van PETEGHEM (2002a) Optimization of solid-phase clean-up prior to liquid chromatographic analysis of ochratoxin A in roasted coffee. *Journal of Chromatography A*, **959**, 327-330.

Stroka, J. and Anklam, E. (2000) Development of a simplified densitometer for the determination of aflatoxins by thin layer chromatography. *Journal of Chromatography A*. **904**, 263.

STROKA, J., ANKLAM, E., van OTTERDIJK, T., 2000, Immunoaffinity column clean-up with one dimensional thin-layer chromatography for the determination of aflatoxins in peanut butter, pistachio paste, fig paste and paprika powder. *Journal of Chromatography,* **83**.

STUDER-ROHR, I, DIETRICH, D. R., SCHLATTER, J. and SCHLATTER, C. (1995) The occurrence of ochratoxin A in coffee – Research Section. *Food and Chemical Toxicology,* **33** (5), 341-355.

STUDER-ROHR, I., DIETRICH, D.R., SCHLATTER, J., SCHLATTER, C. (1994) Ochratoxin A and coffee. *Mitt. Gebiete Lebensmittelunters. Hyg.,* **85,** 719 – 727.

TANIWAKI, M. H., PITT, J. I., TEIXEIRA, A. A., IAMANAKA, B. T. (2003) The source of ochratoxin A in Brazilian coffee and its formation in relation to processing methods. International *Journal of Food Microbiology,* **82**, 173-179.

TERADA, H., TSUBOUCHI, H., YAMAMOTO, K., HISADA, K. and SAKABE, Y. (1986) Liquid chromatography determination of ochratoxin A in coffee beans and coffee products. *Journal of Association of Official Analytical Chemists,* **69**, 960-964.

TRUCKSESS, M. W. (1999) General Referee Reports – Committee on Natural Toxins: Ochratoxin A. *Journal of AOAC International,* **82**, 488-495.

TRUCKSESS, M. W., GILER, J., YOUNG, K., WHITE, K. D., PAGE, S. W. (1999) Determination and survey of ochratoxin A in wheat, barley and coffee – 1997. J*ournal of AOAC International,* **82**, 85-89.

TSUBOUCHI, H., TERADA, H., YAMAMOTO, K., HISADA, K., SAKABE, Y. (1988) Ochratoxin A found in commercial roast coffee. *Journal of Agricultural and Food Chemistry,* **36**, 540-542.

TSUBOUCHI, H., TERADA, H., YAMAMOTO, K., HISADA, K., SAKABE, Y. (1983) Caffeine degradation and increased ochratoxin A production by toxigenic strains of Aspergillus ochraceus isolated from green coffee beans. *Mycopathologia*, **90**, 181-186.

TSUBOUCHI, H., YAMAMOTO, K., HISADA, K., SAKABE, UDAGAWA, S., Y. (1987) Effect of roasting on ochratoxin A level in green coffee beans inoculated with Aspergillus ochraceus. *Mycopathologia,* **97**, 111-115.

TUOMI, T., JOHNSSON, T., HINTIKKA, E-L., REIJULA, K.. (2001) Detection of aflatoxins (G1-2, B1-2), sterigmatocystine, citrinine and ochratoxin A in samples contaminated by microbes. *Analyst*, **126**, 1545-1550.

van der STEGEN, G. V. D., ESSENS, P. J. M., van der LIJN, J. (2001) Effect of roasting conditions on reduction of ochratoxin A in coffee. *Journal of Agric Food Chem*. **49**, 4713-4715.

van der STEGEN, G. V. D., JORISSEN, U., PITTET, A., SACCON, M., STEINER, W., VINCENZI, M., WINKLER, M., ZAPP, J. and SCHALATTER, C., (1997) Screening of European coffee final products for occurrence of ochratoxin A (OTA). *Food Additives and Contaminants,* **14**, 211-216.

VARGAS, E. A., SANTOS, E.A., PITTET, A. (2002) Collaborative Study Submitted for approval by AOAC International: *D-2 Protocol - Determination of ochratoxin A in green coffee by immunoaffinity column clean-up and HPLC.* Ministério da Agricultura e do Abastecimento, Brasil. 28p.

VARGAS, E. A., WHITAKER, T. B., SANTOS, E. A., SLATE, A. B., LIMA, F. B., FRANÇA, R. C. A. (2003) Testing Green Coffee for Ochratoxin A, Part I: Estimation of Variance Components, *Journal of AOAC International*. In press **03096**.

VENTURA, M., VALLEJOS, C., ANAYA, I. A., BROTO-PUIG, F., AGUT, M., COMELLAS, L. (2003) Analysis of ochratoxin A in coffee by solid-phase clean-up and narrow-bore liquid chromatography-fluorescence detector-mass spectrometry. *J. Agric. Food Chem.* **51**, 7564-7567.

WHO – World Health Organization (2001) Safety evaluation of certain mycotoxins in food. Prepared by the 56th meeting of the Joint FAO/WHO Expert Committee on Food Addtives (JECFA), *WHO Food Additives Series* 47, Geneva, 281-415.

SECTION IV
SAMPLE REGISTRATION, PREPARATION AND STORAGE

IV.1. INTRODUCTION

The laboratory must have procedures for sample reception, preparation, packing, storage and disposal and respective records.

The procedures for sample reception and registration aims at assuring identification, confidentiality and traceability of data related to the samples.

The samples received shall be registered after a critical analysis (review) and sent for preparation.

The sample size depends on the sampling plan and/or the size defined by the laboratory. The samples shall be prepared aiming at assuring the smallest particle size and greatest homogenisation possible, representativeness in regard of the original samples received by the laboratory, integrity and preservation.

The packing material utilized for conditioning of samples must be inert and enable the original sample features to be maintained throughout the storage period. The storage conditions must ensure integrity of the sample.

IV.2. REVIEW OF REQUEST, TENDER AND CONTRACT

The sample - duly identified - sent to the laboratory shall be accompanied by the "Term of remittance of sample" or a "Sampling Protocol" (Annex 1) duly filled out according to laboratory specific instructions or a sampling protocol. The term shall contain the necessary information for sample identification and for traceability to a determined lot. If a "Term of Remittance" does not accompany the sample, or if it is not correctly filled out, the client should be notified. In case the sample received is nonconforming with laboratory specific instruction or a sampling protocol, it shall be received upon agreement between the laboratory and the customer.

The sample should not be received if the packing is damaged or if the sample is deteriorated to the extent that the laboratory deems as unacceptable. In this case, the sample should be discarded or replaced by another one, upon agreement with the customer.

Note 1. Any and all observations or definitions related to the samples, other than those predicted in the laboratory procedure should be properly registered in an appropriate registration form field and countersigned by the person in charge and informed to the customer.

IV.3. SAMPLE REGISTRATION

The samples shall be recorded according to the information provided by the customer, contained in the "Term of remittance of Samples" or "Sampling protocol". Recording aims at a unique identification of the sample, assuring its localisation and identification during the period of storage. The samples shall receive a unique code number to represent the year of their registration, origin and sequential entry number in the laboratory.

The use of a controlled registration form or logbook or a computerised system is recommended.

IV.3.1 EXAMPLE OF LACQSA REGISTRATION PROCEDURE

a. Fill out the sample registration form or the sample registration log book;
b. Identify the samples with a code number such as the following example 00XXX00000, being:
   - 00: the two figures referring to the two last figures of the fiscal year (ex.: 2001 = 01);
   - XXX: the letters referring to the code of sample origin;
   - 00000: the five figures referring to the sequential number of the sample's entry at the laboratory (ex: 00001, 00002,...);
c. Write down the sample number, identified by the customer, when informed;
d. Designate the type of matrix;
e. Write down the date of sample registration;
f. Leave in blank the spaces corresponding to the analyses to be performed (as requested by the customer or by the laboratory review registered in the term´s observation field;
g. Specify the customer;
h. Write down in the "Term of remittance of sample " or in the "Sampling Protocol" the Laboratory sample code, the date of entry of the sample at the Laboratory, and pertinent observations;
i. File the "Term of remittance of sample for ochratoxin A analysis";
j. Identify the sample with a label containing the code.

Note 2. When the "registration log book" ends, the person in charge shall write the date and sign the ending page and provide the opening of a new book. Write down on the first sheet the sequential number of the book, by consulting the first page of the latest book ended, the total number of sheets of the book, the date of opening, and sign.

IV.4.SAMPLE PREPARATION

The laboratory, taking in account its facilities, must validate the grinding and homogenisation procedures (Vargas *et al*. 2001).

Caution

Protective clothing, gloves, safety glasses, and earplugs should be worn at all times.

IV.4. 1. EQUIPMENT

a. Air compressor.
b. Blender.
c. IPE -Individual protection equipment.
d. Mixer, industrial mixer and/or helical agitator/mixer.
e. Mills or sample grinder.
f. Refrigerator.
g. Freezer T ≤ - 18 ºC.
h. Vacuum cleaner.
i. Vacuum sealer (optional).
j. Cool Chamber.

IV.4. 2. MATERIAL

a. Inert sample bottles or flasks and/or plastic bags for storage of samples.
b. Sampling scoop and paddles.
c. Paper towel.
d. Utility carriers.
e. Wash bottles, polyethylene or polypropylene screw cap, 500 mL.

IV.4. 3. REAGENTS

a. Sodium hypochlorite solution 1%.
b. Commercial ethanol.
c. Deionised water.

IV.4.4. METHOD

IV.4..4.1. GRINDING

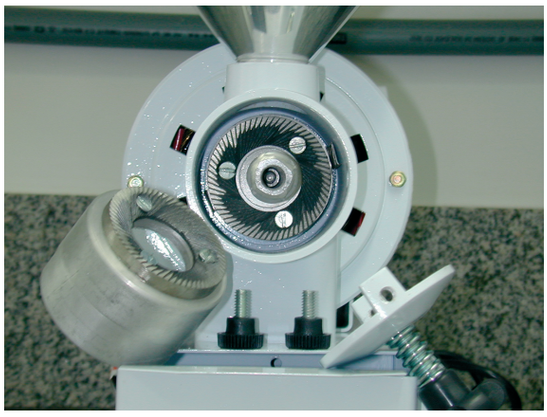
Sample characteristics like hardness, fat and mass content should be taken into account to suit the grinding and homogenization procedures to the equipment available in the Laboratory (MAPA 2003, Vargas *et al*. 2001).

a. Carefully transfer, the green coffee sample to the mill (*Figure 1a, 1b, 1c and 1d*), being sure to avoid loss of content from the package.

b. Grind the green coffee sample to produce a particle size between 18 mesh (1.0 mm) to 35 mesh (0.5 mm) – 80% of the ground sample should be less than 0.5 mm particle size (*Figure 1e and 1f*). The coffee samples should be ground frozen whenever possible.

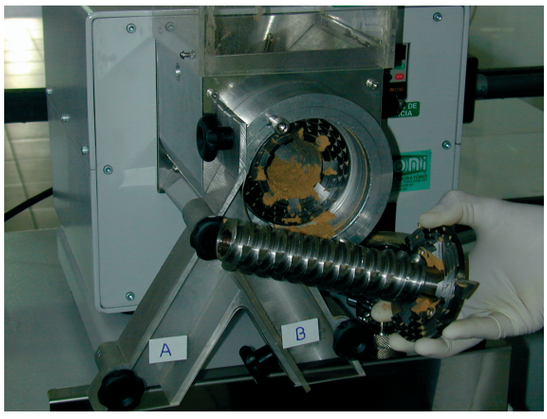
c. Collect the ground sample into a clean dry vessel or package and stick the proper identification with the sample code.

Note 3. After grinding, the green coffee sample should be stored under T ≤ - 18 ºC.



1a

1b



1c

1d

Figures 1a, 1b, 1c and 1d: Types of mills used at LACQSA to grind green coffee samples

1e

1f

Figures 1e and 1f: Particle size: ground green coffee sample

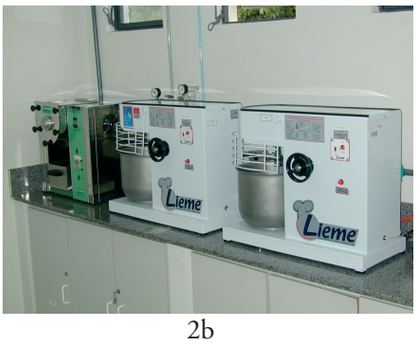
IV.4.4.2. HOMOGENISATION

Homogenisation of coffee samples should be carried out as necessary using different types of homogeniser/mixer or equivalent to obtain appropriate homogenised samples. The laboratory taking into account its facilities must validate the grinding and homogenisation procedures (MAPA 2003, Vargas *et al.* 2001)

The *Figures 2a and 2b* shows the homogenisation at LACQSA (MAPA 2003) of 1 and 5 kg ground coffee samples in a home type mixer and helical agitator/mixer. Ground coffee samples with mass weight above 5kg can be homogenised in an industrial/bakery type mixer for at least 6 hours in 30 min cycles (*Figure 2c and 2d*).

2a

2c



2b

2d

Figures 2a, 2b, 2c and 2d: Type of mixers used to homogenise ground green coffee sample

IV.4.4.3. LABELING, PACKING AND STORAGE

Coffee samples received as beans (i.e., whole, *in natura*) can be stored at ambient temperature, and, preferably under refrigeration (1 to 7ºC) while they await registration and preparation.

a. Stick on the sample package a label containing the Laboratory sample code *(Figure 3a, 3b, 3c).*

b. In case of plastic bags, the label should be stuck internally and the excess of air inside the package should be removed.

c. In case of another type of package, the label should be stuck externally, ensuring that it keeps intact during storage.

The storage of the samples shall be monitored through the specific registration forms and kept preferentially on the door of the refrigerators, freezers and cool chamber.

The samples should be stored at a temperature under T ≤ - 18° C.

3a

3c

3b

Figures 3a, 3b and 3c: (a and b) Packing and labelling (c) a green coffee sample

IV.4.4.4. CLEANING OF THE EQUIPMENT

Decontamination and cleaning of the equipments shall be carried before and after every sample preparation (grinding and homogenization). The pieces of equipment, which are easily disassembled, should be removed and cleaned individually.

Mills, blenders and mixers can be cleaned by removing the excess powder with a vacuum cleaner. Use a brush and needle to release the powder stuck on the parts, aspirate again when necessary. Wash the movable parts of the mill with water and soap, rinse and dry with a towel and/or compressed air.

Figure 4: Cleaning and decontamination of pieces of equipments used at sample preparation

IV.5. DISPOSAL

The samples (including counter-proof) shall be disposed after a period pre-determined by the laboratory from the date of emission of the Analysis Report, according to specific regulation. The date of disposal shall be recorded on the specific registration forms.

The samples should be treated as contaminated material and be handled according to the country's environmental regulation.

IV.6. REFERENCES

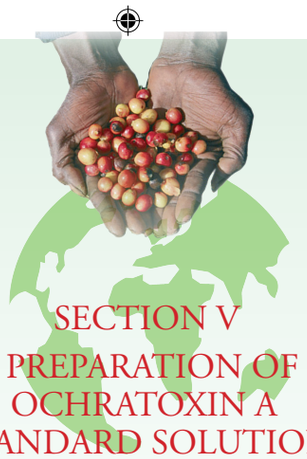
MAPA - Ministério da Agricultura, Pecuária e Abastecimento (2003) LACQSA/LAV-MG - Laboratório de Controle de Qualidade e Segurança Alimentar. *Standard operational procedure (SOP 009 Ed. 06, Rev. 05): Preparation, storage and disposal of samples,* Belo Horizonte, Brasil. 08 p.

MAPA - Ministério da Agricultura, Pecuária e Abastecimento (2001a) LACQSA/LAV-MG - Laboratório de Controle de Qualidade e Segurança Alimentar. *Quality Manual*, *Ed. 02, Rev. 01 and related documentation*, Brasil. 700 p.

MAPA - Ministério da Agricultura, Pecuária e Abastecimento (2001b) LACQSA/LAV-MG - Laboratório de Controle de Qualidade e Segurança Alimentar. *Standard Operational Procedure (SOP 039 ed. 02, rev. 01):* Determination of ochratoxin A In green coffee by immunoaffinity column clean up with LC and TLC, Belo Horizonte, Brasil. 13 p.

VARGAS, E.A., SANTOS, E. A. CASTRO, L. (2001) An analytical approach to assess ochratoxin A contamination in coffee.*19th International Scientific Colloquium on Coffee*. ASIC, 14-18 May, Trieste, Italy. P760.

MAPA - Ministério da Agricultura, Pecuária e Abastecimento (2003) Laboratório de Controle de Qualidade e Segurança Alimentar (2003) *Standard Operational Procedure: Decontamination of glassware (SOP 015 ed 04, rev 03).* Belo Horizonte, Brasil. 7p.



SECTION V
PREPARATION OF OCHRATOXIN A STANDARD SOLUTION

V.1. PRINCIPLE

The ochratoxin *A (*OTA) stock standard solution is prepared with toluene: acetic acid (99 + 1, v/v). The concentration of OTA solution is determined by measurement the absorbance at wavelength of maximum absorption (A) close to λ=333 nm. The real concentration is determined by using measured A and ε = 5440.

This solution should be used to prepare the working and calibration standard solutions.

V.2. EQUIPMENT

a. Air conditioner.
b. Deionizer – 50 or 100 L/h.
c. Analytical balance - capable of weighing down to 0.0001 g.
d. Glass vacuum desiccators.
e. IPE – individual protection equipment.
f. Freezer, T≤-18ºC.
g. Fume hoods.
h. Oven (145 ± 5 ºC).
i. Spectrophotometer - capable of measurements from 200 to 400 nm, with 1 cm quartz cells.
j. Vortex mixer or ultrasonic bath.

V.3. MATERIAL

a. Aluminium foil.
b. Borosilicate glass beakers - 100 and 600 mL.
c. Borosilicate glass Erlenmeyer flask with cap - 125 or 250 and 500mL.
d. Calibrated amber volumetric flasks - 5, 10, 50, 1000, 2000 mL.
e. Calibrated borosilicate glass volumetric flasks – 5, 50 100, 1000, 2000 mL.
f. Calibrated displacement pipettes - 100 - 1000 µL, 20 - 200 µL and 500 - 5000 µL capacity with appropriate tips.
g. Calibrated volumetric pipette 1 and 25 mL.
h. Dropper bulbs.
i. Graduated borosilicate glass cylinder flask - 100 and 1000 mL.
j. Micro reaction vials, amber glass vials - ca. 1.8, 5.0 and 10.0 mL, with cap and septa.

k. Microsyringe – 100 µL.
l. Paper towel.
m. Pasteur pipette.
n. Rubber pear.
o. Silica gel desiccant, or calcium chloride, p.a.
p. Spectrophotometer quartz cell, 10 mm x 10 mm.
q. Stainless steel scoop.
r. Syringe needle.
s. Wash bottles, 500 mL.

V.4. REAGENTS

a. Deionised water.
b. Ethanol, p.a.
c. Glacial acetic acid, p.a.
d. Ochratoxin A standard in form of crystals or dry film, sigma or equivalent.
e. Potassium dichromate ($K_2Cr_2O_7$), primary standard, p.a.
f. Sulphuric acid, p.a.
g. Toluene UV grade.

V.5. CAUTIONS

OTA is a potent nephrotoxin with immunotoxic, teratogenic and potential genotoxic properties. The International Agency for Research on Cancer (IARC) has classified ochratoxin A as a possible human carcinogen (group 2B).

Protective clothing, gloves and safety glasses (*Figure* 1a) should be worn at all times, and all standard solutions and sample preparation stages should be carried out in a fume hood (*Figure* 1b).

1a

1b

***Figure 1:** (a) Individual protection equipment (IPE) (b) Performing OTA analysis in a fume hood*

Swab accidental spills of ochratoxin A with 1% NaOCl bleach, leave for 10 min, and then add 5% aqueous acetone. Rinse all glassware exposed to ochratoxin A with methanol, add 1% NaOCl solution, and after 2h add acetone to 5% of total volume. Let it react for 30 min and then wash thoroughly.

Acetic acid reacts vigorously with strong oxidizers. Wear face shield and heavy gloves when using.

Acetone is highly flammable. It forms explosive peroxides with strong oxidizing agents. Use effective fume hood.

Toluene is toxic. Operations involving this solvent must be performed in a fume hood.

Potassium dichromate is a toxic solid inorganic substance and should be handled with care. Gloves and safety glasses should be worn at all times.

Sulphuric acid always add H_2SO_4 to H_2O. Wear face shield and heavy rubber gloves to protect against splashes. Do not mix with HCl.

Note 1. Disposal of waste solvents must be done according to applicable environmental rules and regulations.

V.6. SOLUTIONS

a. Toluene: acetic acid (99 + 1, v/v).

Add 1 mL of glacial acetic acid to 99 mL of toluene and homogenise.

b. Sulphuric acid solution - 0.009 M.

Pipette 1 mL H_2SO_4 and transfer to 2000 mL volumetric flask containing deionised water.

Complete the volume and homogenise.

c. Potassium dichromate standard solution - approximately 0.250 mM.

Accurately weigh ca 78 mg $K_2Cr_2O_7$ primary standard (previously desiccated at 140-150^{0}C for 30-60 minutes), quantitatively transfer to 1000 mL volumetric flask and complete the volume with 0.009 M H_2SO_4 solution and homogenise.

Calculate molarity to 3 significant figures (MW $K_2Cr_2O_7$ = 294.2) using the Equation 1.

Equation 1

$$mM = \frac{W(g) \times 1000}{MW}$$

Where:

mM = concentration of potassium dichromate standard solution;

W = weight of $K_2Cr_2O_7$ (g);

MW = molecular weight of $K_2Cr_2O_7$.

Note 2. If exactly 78 mg of $K_2Cr_2O_7$ primary standard is weighed, the solution concentration is 0.265 mM. This value should be used to prepare 0.132 and 0.0662 mM $K_2Cr_2O_7$ solutions.

d. Approximately 0.125 mM potassium dichromate standard solution.

Pipette 25 mL 0.250 mM $K_2Cr_2O_7$ solution to 50 mL volumetric flask. Complete the volume with 0.009 M H_2SO_4 solution and homogenise.

e.Approximately 0.0625 mM potassium dichromate standard solution.

Pipette 25 mL 0.125 mM $K_2Cr_2O_7$ solution to 50 mL volumetric flask. Complete the volume with 0.009 M H_2SO_4 and homogenise.

f. Ochratoxin A stock standard solutions (approximately 40 µg/mL)

Use label statement of ochratoxin A standard as guide. Introduce a known volume of toluene: acetic acid (99 + 1, v/v) into a septum-capped vial containing solid standard of ochratoxin A using a microsyringe.

Agitate using a vortex mixer, and then cautiously, transfer an aliquot of standard to give a concentration of 40 µg/mL approximately, to a volumetric flask, complete the volume with toluene: acetic acid (99 + 1, v/v), and homogenize using vortex mixer and/or ultrasonic bath. Determine the concentration of ochratoxin A standard solution by measuring the absorbance (A) using a spectrophotometer (*Figure* 2).

Figure 2: *Spectrophotometer used for determination of OTA standard solution concentration*

Prior to determining the ochratoxin A concentration, the spectrophotometer should be checked with potassium dichromate standard solutions.

g. Working standard solution (approximately 1 µg/mL).

Prepare ochratoxin A working standard solution by transferring an appropriate aliquot of the stock standard solution (approximately 40 µg/mL) into a volumetric flask and diluting with toluene: acetic acid (99 + 1, v/v). This solution should be used for spiking and to prepare the LC and TLC calibration solutions.

Warning: Ochratoxin A standard solutions must be stored in a freezer under T ≤ -18° C.

V.7. CHECKING THE SPECTROPHOTOMETER

Transfer the aliquots of 0.250, 0.125 and 0.0625 mM potassium dichromate standard solutions to the spectrophotometer cell and record the UV spectrum by measuring the absorbance (A) at maximum absorption between 350 - 365 nm against 0.009 M sulphuric acid solution as blank solvent.

Calculate the molar absorptivity (ε) at each concentration of potassium dichromate standard solution by using the Equation 2.

Equation 2

$$\varepsilon = \frac{(A \times 1000)}{mM}$$

Where:

A = absorbance of potassium dichromate standard solutions;

mM = concentration of potassium dichromate standard solutions.
Average 3 ε to obtain ε.

Determine the correction factor (CF) for the instrument by using the Equation 3.

Equation 3

$$CF = \frac{3160}{\varepsilon_m}$$

Where 3160 is the value for ε of potassium dichromate standard solutions.

If CF < 0.95 or CF >1.05, check either the instrument or the potassium dichromate standard solution to determine and eliminate the cause.

V.8. DETERMINATION OF OCHRATOXIN A CONCENTRATION

Transfer an aliquot of ochratoxin A stock standard solution (approximately 40 µg/mL) to a spectrophotometer cell and record the UV spectrum (*Figure* 3) of ochratoxin A solution by measuring the absorbance (A) at wavelength of maximum absorption close to 333 nm against toluene: acetic acid (99 + 1, v/v). Determine the exact concentration of the solution by using the Equation 4.

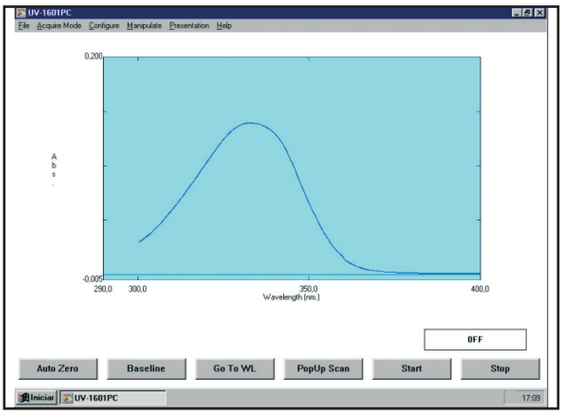


Figure 3: *UV spectrum of OTA standard solution in toluene: acetic acid (99 + 1, v/v).*

Equation 4

$$OTA_{\mu g/mL} = \frac{(A \times MW \times 1000)}{\varepsilon_1}$$

Where:

OTA (µg/mL) = ochratoxin A concentration;

MW (403.8): ochratoxin A molecular weigh;

ε (5440): molar absorptivity of ochratoxin A in toluene: acetic acid (99 + 1, v/v);

A: absorbance of the ochratoxin A standard solution at maximum absorption (λ ca. 333 nm).

V.9. REFERENCE

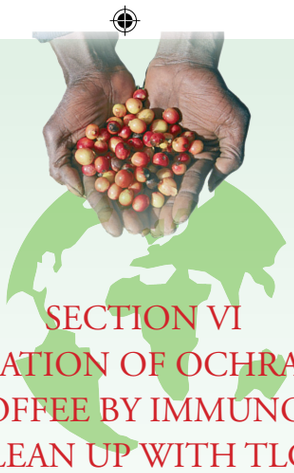
AOAC – Association of Official Analytical Chemist (1998) Natural Toxins. *Official Methods of AOAC International*. 49, 39-40. 16th edition, 4th revision. (Software Adobe and E-DOC/CJS).

AOAC – Association of Official Analytical Chemist (2000) Natural Toxins. *Official Methods of AOAC International*. 2, 49, 1-64 and Appendix A. 17th edition. Edited by William Horwitz.

MAPA - Ministério da Agricultura, Pecuária e Abastecimento (2001) LACQSA/LAV-MG - Laboratório de Controle de Qualidade e Segurança Alimentar. *Standard Operational Procedure (SOP 039 ed. 02, rev. 01):* Determination of ochratoxin A In green coffee by immunoaffinity column clean up with LC and TLC, Belo Horizonte, Brasil. 13 p.

MAPA - Ministério da Agricultura, Pecuária e Abastecimento (2001a) LACQSA/LAV-MG - Laboratório de Controle de Qualidade e Segurança Alimentar. *Standard Operational Procedure (SOP 013 ed. 04, rev. 03*): Preparation of mycotoxin standard solutions, Belo Horizonte, Brasil, 07 pp.

TRUCKSESS, M. W. (1999) General Referee Reports – Committee on Natural Toxins: Ochratoxin A. *Journal of AOAC International*, 82, 488-495.



SECTION VI
DETERMINATION OF OCHRATOXIN A IN GREEN COFFEE BY IMMUNOAFFINITY COLUMN CLEAN UP WITH TLC AND LC

VI.1. PRINCIPLE

A test portion is extracted with methanol: 3% aqueous sodium hydrogen carbonate solution (1+1, v/v). The extract is filtered, diluted with phosphate buffered saline (PBS), and applied to an immunoaffinity column containing antibodies specific for ochratoxin A (OTA). After washing the toxin is eluted from the column with methanol, separated (reversed phase column C18), detected (fluorescence detector) and quantified by liquid chromatography or thin layer chromatography.

VI.2. EQUIPMENT

a. Air conditioner.

b. Air pump.

c. Deionizer – 50 or 100 L/h.

d. Scale - capable of weighing down to 0.01g.

e. IPE – individual protection equipment.

f. Freezer, T ≤ -18ºC.

g. Fume hoods.

h. Homogeniser/Blender.

i. Refrigerator.

j. Water bath or block heater - capable of maintaining temperature at 40 to 45 °C with gas flow adapter.

k. Timer.

l. Ultrasonic bath.

m. Vacuum manifold.

n. Vacuum/pressure pump.

o. Vortex mixer.

p. LC apparatus comprising the following:

- Valve injection system - 20 µL loop size;
- Mobile phase pump capable of pumping 1 mL/minute with negligible pulsation;
- Fluorescence detector capable to provide λ = 332 nm excitation and λ = 476 nm emission wavelengths;
- Computer based data processing system;
- Reversed Phase (C18) column 250x4.6 mm with 5 µm particles;
- (C18) Guard column;
- Degasser.

q. Cromatoviewer UV light, λ=365 nm.

r. Densitometer, wavelength scanning range 200-700 nm, measurement range: 200-650 nm, with mercury lamp, mode: fluorescence.

s. Microsyringe – 20 µL, Hamilton type or equivalent.

t. TLC developing tank, holding two 10x20 cm plates.

VI.3. MATERIAL

a. 10 cm diameter glass funnels with short stem (7 cm).

b. Adjustable clamp.

c. Aluminium foil.

d. Bond elut adapters to fit 1.3 and 6 mL tubs, Varian type or equivalent.

e. Bond-elut Luer stopcock, Varian or equivalent.

f. Borosilicate glass 250-500 mL and 1000 mL filtering flasks (glass side arm).

g. Borosilicate glass or polypropylene beakers - 100, 250, 600 and 1000 mL.

h. Borosilicate glass Erlenmeyer flask, with cap, 125 or 250 mL.

i. Borosilicate glass volumetric flask - 100, 500, 1000 and 2000 mL.

j. Calibrated borosilicate glass volumetric pipettes, 1 and 4 mL.

k. Calibrated micropipettes - 100 - 1000 µL, 20 - 200 µL and 500 - 5000 µL capacity with appropriate tips.

l. Disposable syringe barrels - to be used as reservoirs (70 mL capacity) luer lock.

m. Dropper bulbs.

n. Fibreglass membrane Whatman GF/B 1 µm, ∅55 mm or equivalent.

o. Membrane 0.45 µ?m for aqueous organic solvent.

p. Filtration System – with funnel top, funnel membrane support 47 mm, aluminium clamp, stopper and side-arm flask. Büchner funnels, for ∅55 mm glass filter can be used.

q. Folded fast qualitative filter paper, Whatman number 4, ∅24 cm or equivalent.

r. Glass borosilicate syringe – with embolus (10 mL capacity), needleless.

s. Graduated borosilicate glass cylinder flask - 10, 50, 100, 250 and 1000 mL.

t. Immunoaffinity columns.

➢ The immunoaffinity column should contain antibodies raised against ochratoxin A. The column should have a maximum capacity of not less than 100 ng of ochratoxin A and give a recovery of not less than 85% when ochratoxin A standard in methanol: 3% sodium bicarbonate (1 + 1, v/v)/ PBS solution (4 + 96, v/v) is passed through.

u. Latex tubes 0.8 cm internal diameters.

v. Micro reaction vials, amber glass vials - ca. 2.0, 5.0 and 10 mL, with cap and septa.

w. Parafilm.

x. Pasteur pipettes.

y. Polypropylene centrifuge tubes with screw caps, 15 mL or test tubes with cap to collect a 4 mL volume.

z. Ring-like clamp for 7 cm diameter short stem glass funnels.

aa. Rubber pear.

bb. Sampling scoop, polypropylene (25 g).

cc. Scissors.

dd. Paper towel.

ee. Pipette racks.

ff. Pre-coated silica gel 60 (normal) TLC glass plate, 10x10 or 10x20 or 20x20 cm, 0.25 mm thickness, without fluorescent indicator.

gg. Utility carriers.

hh. Polyethylene or polypropylene wash bottles, 500 mL.

VI.4. REAGENTS

Unless otherwise specified, use only reagents of a recognised analytical grade and/or HPLC grade.

a. 88% formic acid, p.a.

b. Acetonitrile, HPLC grade.

c. Anhydrous disodium hydrogen phosphate (Na_2HPO_4), p.a.

d. Commercial ethanol.

e. Deionised water.

f. Detergent extran.

g. Ethanol, p.a.

h. Ethyl acetate, p.a.

i. Glacial acetic acid, p.a.

j. Helium-purified compressed gas or other degassing system.

k. Methanol, HPLC grade.

l. Methanol, p.a.

m. Nitrogen gas (N_2) (purity > 99.9%).

n. Potassium chloride (KCl), p.a.

o. Potassium dihydrogen phosphate (KH_2PO_4), p.a.

p. Silicone, p.a.

q. Sodium chloride (NaCl), p.a.

r. Sodium hydrogen carbonate ($NaHCO_3$), p.a.

s. Toluene (UV grade).

VI.5. CAUTIONS

Ochratoxin A is a potent nephrotoxin with immunotoxic, teratogenic and potential genotoxic properties. The International Agency for Research on Cancer (IARC) has classified ochratoxin A as a possible human carcinogen (group 2B).

Protective clothing, gloves and safety glasses (*Figure* 1a) should be worn at all times, and all standard and OTA analysis should be carried out in a fume hood (*Figure* 1b).

1a

1b

Figure 1: (a) Individual protection equipment (IPE) (b) Performing OTA analysis in a fume hood

Swab accidental spills of ochratoxin A with 1% NaOCl bleach, leave for 10 min, and then add 5% aqueous acetone. Rinse all glassware exposed to ochratoxin A with methanol, add 1% NaOCl solution, and after 2h add acetone to 5% of total volume. Let it react 30 min and then wash thoroughly.

Toluene is toxic. Operations involving this solvent must be performed in a fume hood.

Methanol is hazardous, and the samples must be blended using an explosion proof blender housed within a fume hood. All analyses should be carried out inside the fume hood.

Disposal of waste solvents must be done according to applicable environmental rules and regulations.

VI.6. SOLUTIONS

a. Ochratoxin A working standard solutions

Prepared according to Section V Preparation of Ochratoxin A Standard Solution.

b. 3% aqueous sodium hydrogen carbonate solution

Dissolve 30 g of sodium hydrogen carbonate using deionised water and transfer to 1000 mL borosilicate glass volumetric flask. Complete the volume with deionised water and homogenise

c. Methanol: 3% aqueous sodium hydrogen carbonate solution (1 + 1, v/v)

Add 1000 mL of 3% aqueous sodium hydrogen carbonate solution to 1000 mL of methanol and homogenise.

d. Phosphate buffered saline (PBS) solution pH ~7.0

Dissolve 0.20 g of potassium dihydrogen phosphate, 1.10 g of anhydrous disodium hydrogen phosphate, 8.00 g of sodium chloride, 0.20 g of potassium chloride using deionised water and transfer to 1000 mL borosilicate glass volumetric flask. Complete the volume with deionised water and homogenise.

e. Aqueous glacial acetic acid solution (29 + 1, v/v)

Add 30 mL of glacial acetic acid to 870 mL of deionised water and filter in a 0.45 m?m membrane.

f. LC mobile phase - acetonitrile: methanol: aqueous glacial acetic acid solution (29 + 1, v/v) (35 + 35 + 30, v/v/v)

Add 350 mL of methanol and 350 mL of acetonitrile to 300 mL of aqueous glacial acetic acid solution (29 + 1, v/v) and homogenise. This mobile phase should be degassed by ultrasonication or other degassing system and during pumping by bubbling helium into the mobile phase reservoir.

g. LC calibration standard solutions (1.0 - 40 ng/mL)

Pipette 200 µL of diluted working standard solution (approximately 1000 ng/mL) into a 5 mL volumetric flask. Evaporate the solution just to dryness under a stream of nitrogen at room temperature. Re-dissolve with 5000 µL of LC mobile phase to give a concentration of 40 ng/mL (standard 1). Then use this solution (standard 1) to prepare 2000 µL of calibrant solutions (standard 2 to standard 6), as indicated in Table 1.

Table 1: LC calibration standard solutions for OTA analysis

Calibrant solution	*Volume of solution (standard 1) (µL)*	*Volume of LC mobile phase (µL)*	*Resulting OTA concentration (ng/mL)*	*Resulting OTA sample contamination (ng/g)*
Standard 1	-	-	40	24.0
Standard 2	1500	500	30	18.0
Standard 3	1000	1000	20	12.0
Standard 4	500	1500	10	6.0
Standard 5	250	1750	5	3.0
Standard 6	50	1950	1	0.6

h.TLC calibration standard solutions (2.5 - 60 ng/mL)

Pipette 300 µL of diluted working standard solution (approximately 1000 ng/mL) into a 5 mL volumetric flask. Complete the volume with toluene: acetic acid (99 + 1, v/v) to give a concentration of 60 ng/mL (standard 1). Then use this solution (standard 1) to prepare 2000 µL of calibrant solutions (standard 2 to standard 7), as indicated in Table 2.

Table 2: TLC calibration standard solutions for OTA analysis

Calibrant solution	*Volume of solution (standard 1) (µL)*	*Volume of toluene:acetic acid (99 + 1, v/v) added*	*Resulting OTA concentration (ng/mL)*	*Resulting OTA sample contamination (ng/g)*
Standard 1	-	-	60	10.0
Standard 2	1330	670	40	8.0
Standard 3	1000	1000	30	6.0
Standard 4	670	1330	20	4.0
Standard 5	335	1665	10	2.0
Standard 6	165	1835	5	1.0
Standard 7	85	1915	2.5	0.5

Warning: Ochratoxin A standard solution in toluene:acetic acid (99 + 1, v/v) must be stored in a freezer under T ≤ -18 °C .

Note 1. Use amber flask or protect the flasks against the light using an aluminium foil.

i. Water: ethanol (10 + 2, v/v).

Add 20 mL of ethanol to 100 mL borosilicate glass volumetric flask. Complete the volume with deionised water and homogenise.

i. Ethanolic sodium hydrogen carbonate solution.

Weigh exactly 6 g of sodium hydrogen carbonate, transfer quantitatively with water: ethanol (10 + 2, v/v) solution to a 100 mL volumetric flask, complete the volume and homogenise.

k. Toluene: acetic acid (99 + 1, v/v).

Add 1 mL of glacial acetic acid to 99 mL of toluene and homogenise.

l. Toluene: ethyl acetate: 88% formic acid (6 + 3 + 1, v/v/v).

Add 10 mL of formic acid and 30 mL of ethyl acetate to 60 mL of toluene and homogenise.

VI.7. PREPARATION OF TEST PORTION

VI.7.1. SAMPLE EXTRACTION

a. Weigh, to the nearest 0.10 g, 25 g test portion of green coffee sample, at room temperature, into a flask (*Figures* 2a and 2b).

2a

2b

b. Add 200 mL of methanol: 3% aqueous sodium hydrogen carbonate solution (*Figures* 3a and 3b). Blend for 5 min with a homogeniser (*Figures* 3c and 3d).

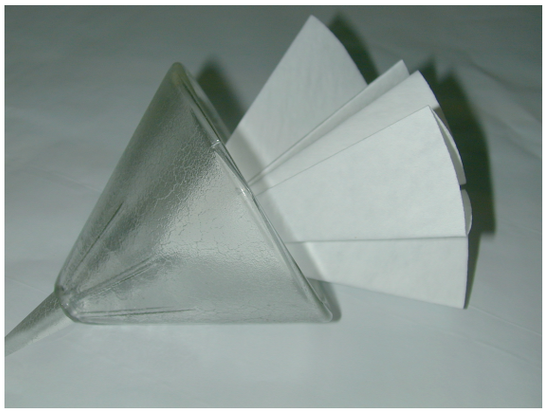
3a

3b

3c

3d

c. Immediately following blending, filter the mixture through a folded qualitative paper filter (*Figures* 4a, 4b and 4c).

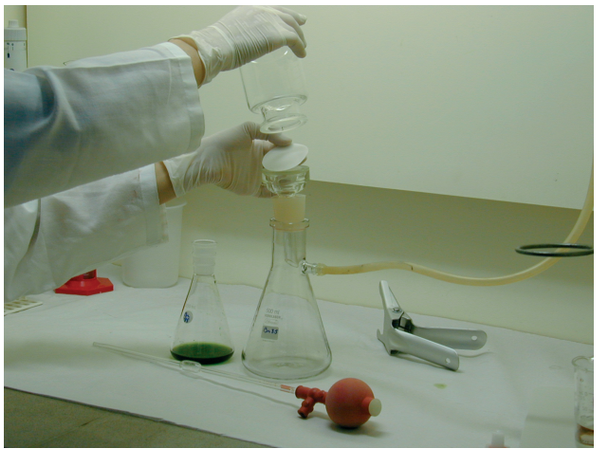


4a

4b

4c

d. Immediately following filtration, collect the filtrate and re-filter through a fibreglass membrane, Whatman GF/B 1µm, ∅55 mm, using a vacuum system (*Figures* 5a and 5b).



5a

5b

e. Immediately following the second filtration, take an aliquot of 4 mL of filtered extract (*Figure* 6a) and transfer to a 100 mL graduated cylinder or volumetric flask (*Figures* 6b and 6c).

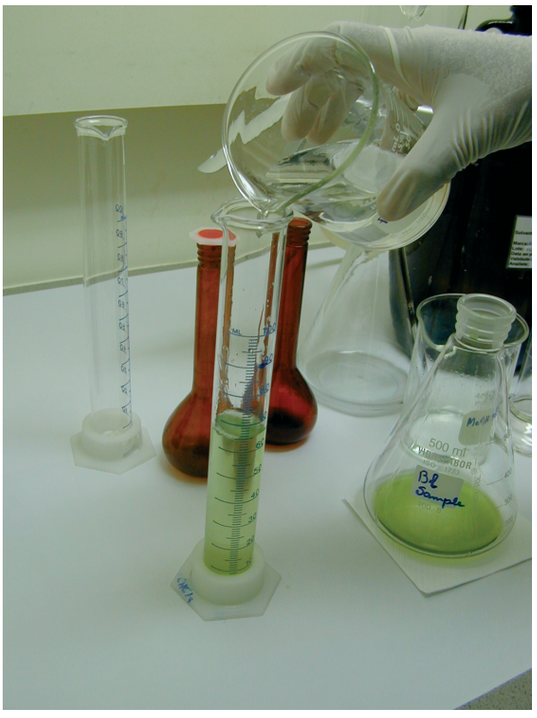
6a

6b

6c

f. Dilute to 100 mL with phosphate buffered saline (PBS) and homogenise (*Figures* 7a and 7b).

7a

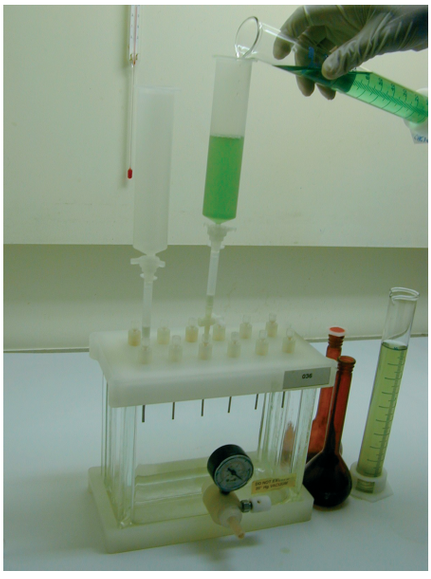


7b

VI.7.2. IMMUNOAFFINITY COLUMN CLEAN-UP

a. Connect the immunoaffinity column to the vacuum manifold and attach a reservoir of 60 mL capacity to the immunoaffinity column.

b. Transfer the 100 mL of diluted sample extract to the reservoir and pass through the immunoaffinity column at flow rate of 2-3 mL/min. Do not exceed the flow rate of 3 mL/min and do not allow the column to dry up. Let it pass by gravity or pushing down slightly with an embolus or applying a little vacuum (*Figures* 8a and 8b).



8a

8b

c. Wash the column with 10 mL of deionised water at flow rate of 3 mL/min (*Figure* 8c).

8c

d. Dry the column by applying a little vacuum for 30 seconds or apply slight pressure by pushing down the embolus.

VI.7.3. OCHRATOXIN A ELUTION

a. Disconnect the immunoaffinity column and replace the 60 mL reservoir with a 10 mL glass syringe.

b. Apply 4 mL methanol to the 10 mL glass syringe and wait for 3 minutes to allow the methanol to permeate the gel, prior to elution (*Figure* 9a).

c. Elute the ochratoxin A from the column into a centrifuge or test tub at flow rate of 2 - 3 mL/min using positive pressure (*Figure* 9b).

d. Evaporate the eluate to dryness using a gentle stream of nitrogen in a water bath or block heater at 40 – 45 ºC (*Figure* 9c).

9a

9b

9c

VI.7.4. SEPARATION, DETECTION AND QUANTIFICATION OF OCHRATOXIN A BY LC ANALYSIS

Prepare a calibration curve by injecting 20 µL of 1, 5, 10, 20, 30 and 40 ng/mL (Table 1) LC standard solutions at the beginning of the analysis. Plot the peak area against the mass of injected ochratoxin A and check the curve for linearity. The linearity of the standard calibration curve should not be less than $r^2 \geq 0.99$ (*Figures* 10a and 10b).

VI.7.4.1. LC CONDITIONS

- Loop: 20 µL.
- Temperature of injection: column temperature.
- Column temperature: room temperature.
- Elution flow rate: 0.8 mL/min.
- Mobile phase: Acetonitrile: methanol: aqueous glacial acetic acid solution (29 + 1, v/v) (35 + 35 +

30, v/v/v).

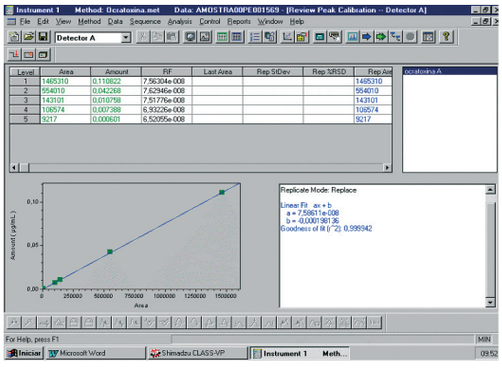
Inject aliquots of 20 µL of the test solutions into the chromatograph in the same conditions used for preparation of the LC calibration standard curve. Identify the peak of ochratoxin A of the test solution (*Figure* 10c) by comparing the retention time of the sample with that of the LC standard solutions. If the reading of the samples is higher than the standard, dilute the samples and re-inject.

Re-dissolve the residue obtained in the ochratoxin A elution step with 300 µL of acetonitrile: methanol: aqueous glacial acetic acid solution (29 + 1, v/v) (35 + 35 + 30, v/v/v*)* and homogenise in a vortex mixer and/or in ultrasonic bath.

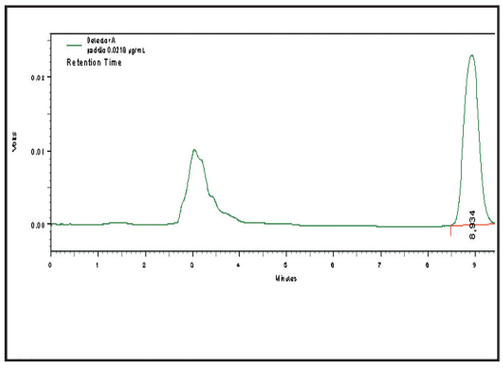
Ochratoxin A peak should be completely separated from any interfering peak, with retention time of approximately 10 min when a reversed phase (C18) column 250x4.6 mm with 5 µm particles and mobile phase acetonitrile: methanol: aqueous glacial acetic acid solution (29 + 1, v/v) (35 + 35 + 30, v/v/v) are employed (*Figure* 10c).

10a

10b



10c



VI.7.4.2 CALCULATION OF OCHRATOXIN A CONTAMINATION - LC ANALYSIS

Determine from the calibration standard curve, the mass in ng of the ochratoxin A in the aliquot of test solution injected onto the LC column.

Calculate the concentration of ochratoxin A (ng/g) using:

Equation 1

$$\text{OTA}\,(\text{ng/g}) = \frac{M_{ota}(ng)}{W(g)} = \frac{M_{ota} \times V_1 \times V_3}{M_s \times V_2 \times V_4} = \frac{M_{ota}(ng)}{0.0333g}$$

Where:

M_{OTA} = mass of OTA (ng) in the aliquot of extract (20 µL) injected into LC

W = equivalent weight of test portion injected into the LC system (0.0333 g)

M_s = mass of test portion (25 g)

V_1 = volume of extraction solution (200 mL)

V_2 = volume of filtrate loaded onto the immunoaffinity column (4 mL)

V_3 = volume of LC mobile phase used for taking up the dry residue (300 µL)

V_4 = volume of extract injected onto the LC column (20 µL)

VI.7.5 SEPARATION, DETECTION AND QUANTIFICATION OF OCHRATOXIN A BY ONE-DIMENSIONAL TLC

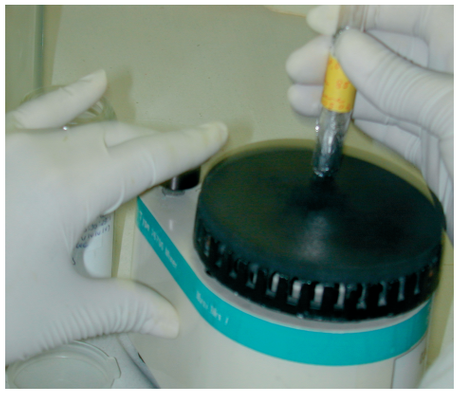
a. Re-dissolve the residue obtained in the ochratoxin A elution step with 100 µL of toluene: acetic acid (99 + 1, v/v) and homogenise in vortex mixer and/or in ultrasonic bath (*Figure* 11a).

b. Spot 20 µL of extract and OTA standard solutions (Table 2) on a TLC plate (*Figure* 11b) at 15 mm from both right and left edges, and from the bottom edge of the plate, maintaining 10 mm intervals, according to the spotting scheme shown in *Figure* 12.

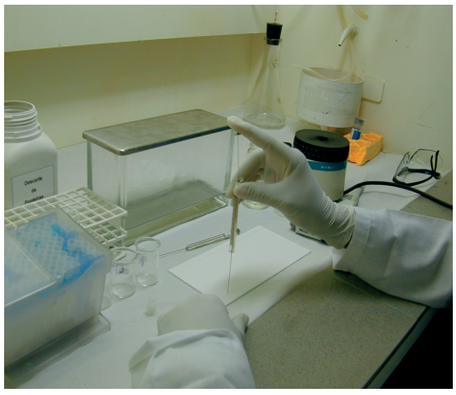
c. Elute the TLC plate with toluene: ethyl acetate: 88% formic acid (6 + 3 + 1, v/v/v) in an unsaturated tank (*Figure* 11c) until the solvent front reaches ca. 10 mm from the upper edge of the plate.

d. Dry the TLC plate in fume hood for at least 5 minutes before quantification by visual and/or densitometer.

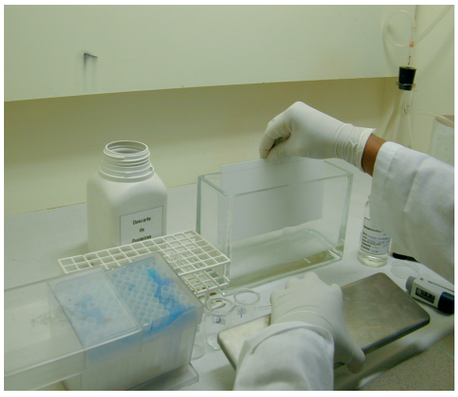
Note 2. Always dry the TLC plate before exposure to UV light. UV light from sunlight or fluorescent lamps can catalyse changes in compounds being examined when exposed to adsorbent surface (TLC plate), particularly in the presence of solvent.



11a



11b



11c

Figure 11: Determination and quantification of OTA step comprising (a) Dissolution and homogenisation of extract using a vortex mix (b) Application of OTA standard solution and green coffee sample on TLC plate (c) Elution of OTA using an unsaturated tank containing toluene: ethyl acetate: 88% formic acid (6 + 3 + 1, v/v/v)

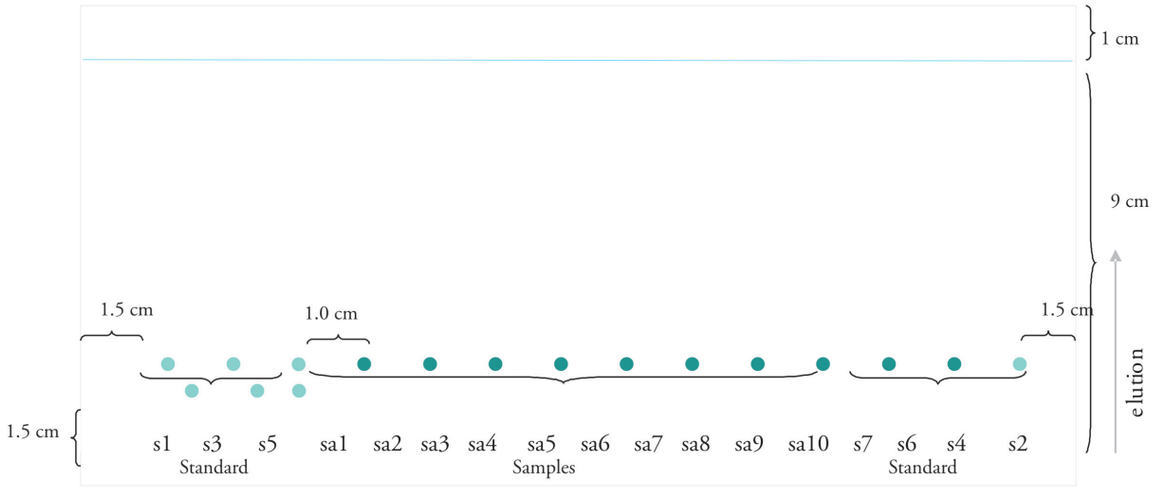
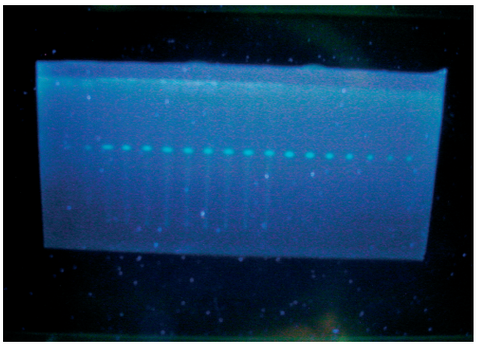


Figure 12: Schematic representation of a one-dimensional thin layer chromatography, containing OTA standard solutions and green coffee samples

VI.7.5.1. VISUAL ANALYSIS

Compare by visual examination under ultraviolet light cromatoviewer at 365 nm (*Figure* 13a) the fluorescence intensity of the samples with those of OTA standard solution spotted on a TLC plate. Make the quantification by comparing the fluorescence intensity of the samples and standard as "equal to (=), smaller than (<), between (-) or higher than (>)" the correspondent standard – see *Figure* 13b as an example of OTA on a silica gel TLC plate after elution with toluene: ethyl acetate: 88% formic acid (6 + 3 + 1, v/v/v).

13a



13b

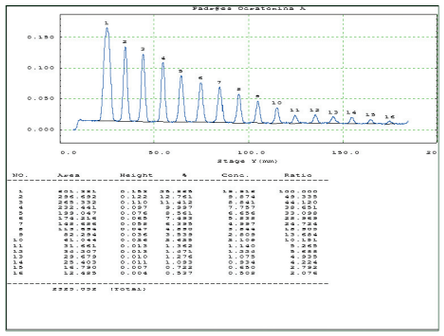
Figure 13: (a) UV light cromatoviewer (b) Silica gel 60 TLC plate after development in toluene: ethyl acetate: 88% formic acid (6 + 3 + 1, v/v/v) as visualised under 365 nm UV light: standard and spiked sample extract

VI.7.5.2. DENSITOMETRIC ANALYSIS

Scan the plate using a densitometer (*Figure* 14a) and plot the peak area against the mass of ochratoxin A standard solution spotted on TLC plate and check the linearity of the calibration curve (*Figures* 14b and 14c).

14a

14b



14c

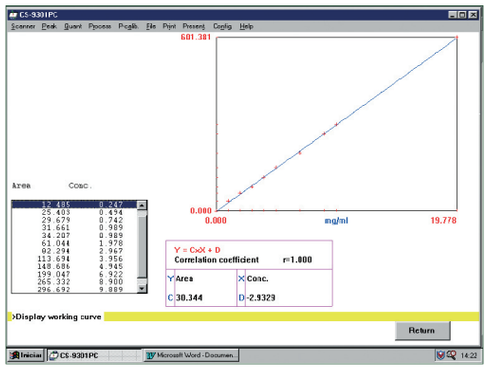


Figure 14: (a) Densitometer (b) Chromatograms of OTA standard solution in toluene: acetic acid (99:1, v/v) spotted on normal TLC silica gel 60 (c) Calibration curve of OTA standard solution (0.5 to 20.0 ng/mL)

Densitometer, 200-700 nm.

- mercury lamp;
- mode: fluorescence;
- λ = ~ 324 nm;

The linearity of the standard calibration curve should not be less than r^2>0.98. Compare the area of chromatographic peak of the samples with those of the standard calibration curve.

In case the area of the samples is not within the range of the calibration curve, the sample extract should be quantitatively diluted and re-spotted.

VI.7.5.3. OCHRATOXIN A **CONFIRMATION**

The OTA contamination is confirmed by spraying the TLC plates with ethanolic sodium hydrogen carbonate solution, drying the plate and observe the fluorescence under UV light (365 nm). The OTA greenish fluorescence should change to bluish.

VI.7.5.4. **CALCULATION OF** OCHRATOXIN A **CONTAMINATION - TLC ANALYSIS**

- Visual quantification

Equation 2

$$OTA(ng/g) = \frac{SC \times V_1 \times V_3 \times V_5}{V_2 \times M_S \times V_4}$$

- Densitometric quantification

Equation 3

$$OTA(ng/g) = \frac{(M_{OTA} \times V_1 \times V_3)}{V_2 \times M_S \times V_4}$$

Where:

OTA (ng/g) = concentration of OTA determined in the sample
SC = OTA standard concentration (µg/mL)
M_s = mass of test portion (g)
M_{OTA} = mass of OTA present in the aliquot spotted on TLC plate (ng)
V_1 = volume of extraction solution (200mL)
V_2 = volume of filtrate loaded onto the immunoaffinity column (4mL)
V_3 = volume of solution used for taking up the dry residue (100µL)
V_4 = volume of extract spotted on TLC plate (µL)
V_5 = volume of standard to which the sample fluorescence was compared (µL)

Note 3: Use sodium hypochlorite 1% solution to decontaminate OTA residues and all material exposed to ochratoxin A.

VI.8. PERFORMANCE OF THE METHOD

VI.8.1. LIQUID CHROMATOGRAPHY

The in-house performance characteristics of the method, determined by recovery test with spiked green coffee samples are shown in Table 3. The performance characteristics of the method obtained in a collaborative study with 18 participants from different countries are shown in Table 4.

Table 3: In-house method characteristics for OTA determined with spiked green coffee samples

Parameters	*Results*	*Acceptability*
Range (ng/g)	0.20 a 109.2	-
Recovery (%)	80.0 a 107.9	Satisfactory
Relative standard deviation (%RSD)	10.7 a 21.1	Satisfactory
Linearity (r^2)	> 0.9 (range 0.20 a 60.0 ng/g)	Satisfactory
Limit of quantification	0.20 ng/g	-
Limit of detection	0.12 ng/g	-

The repeatability (n=24) of the method in the analysis of a naturally contaminated green coffee samples (5.15 + 0.60 ng/g) was 11.6%.

Table 4: Statistical analysis of collaborative study lresults for ochratoxin A from 18 laboratories - performance characteristics

Sample	*Average (ng/g)*	*Mean Recovery (%)*	*r*	S_r	RSD_r *(%)*	*R*	S_R	RSD_R *(%)*
Blanc	nc	-	nc	nc	nc	nc	nc	nc
Spiked	4.48	112.4	0.93	0.33	7.42	2.05	0.73	16.34
Nat-1	2.60	-	1.22	0.44	16.78	1.50	0.53	20.51
Nat-2	6.32	-	3.70	1.32	20.94	5.16	1.84	29.17
Nat-3	12.89	-	3.33	1.19	9.24	7.30	2.60	21.15

nc: Statistical parameters not calculated; level were below limit of detection.

Nat: Naturally contaminated sample.

VI.8.2.THIN LAYER CHROMATOGRAPHY

The characteristics of the TLC method - recovery, in-house repeatability (RSD) were assessed by means of recovery tests with spiked samples in the range of 1.8 to 109 ng/g (6 levels of contamination, n=3) (Table 5). The mean recoveries of OTA spiked samples (1.8 – 109 ng/g) are 98.4 and 103.8% for densitometry and visual analysis, respectively. The relative standard deviations for densitometric and visual analysis vary from 1.1 to 24.9%, and from 0.0 to 18.8% respectively.

Table 5: In-house method characteristics for OTA determined with spiked green coffee samples (triplicate of analysis, triplicate of application) by normal TLC with densitometry

Parameters	*Results*	*Acceptability*
Range (ng/g)	1.8 to 109	-
Recovery (%)	84 to 133	Satisfactory
Relative standard deviation (%RSD)	1.1 to 25	Satisfactory
Linearity (r^2) (by densitometer)	> 0.9 (range 0.04 to 84 ng)	Satisfactory
Limit of quantification	0.5 ng/g	-
Limit of detection	0.5 ng/g	-

VI.9. REFERENCES

BRASIL (2000) Métodos de Referência para análise de ocratoxina A em café verde, *Brazilian Official Daily Journal (DOU, Diário Oficial da União)*, Instrução Normativa SDA, nº. 09, 24/03/2000, seção 1, 35-41.

CEN - European Committee for Standardisation, (1999) *CEN Report: Food Analysis*. Biotoxins: Criteria of analytical methods of mycotoxins, CR 13505:1999 E. Brussels. 8 p.

MAPA - Ministério da Agricultura, Pecuária e Abastecimento (2001) LACQSA/LAV-MG - Laboratório de Controle de Qualidade e Segurança Alimentar. *Quality Manual*, *Ed. 02, Rev. 01 and related documentation*, Brasil. 700 p.

MAPA - Ministério da Agricultura, Pecuária e Abastecimento (2001a) LACQSA/LAV-MG - Laboratório de Controle de Qualidade e Segurança Alimentar. *Standard Operational Procedure (SOP 039 ed. 02, rev. 01):* Determination of ochratoxin A In green coffee by immunoaffinity column clean up with LC and TLC, Belo Horizonte, Brasil. 13 p.

PITTET, A., TORNARE, D., HUGGET, A., VIANI, R. (1996) Liquid chromatographic determination of ochratoxin A in pure and adultered soluble coffee using an immunoaffinity column cleanup procedure. *Journal of Agricultural Food Chem*. 44, 3564-3569.

SANTOS, E. A., VARGAS, E. A. (2002) Immunoaffinity column clean up and thin layer chromatography for determination of ochratoxin A in green coffee, *Food Additives and Contaminants*, 19, nº. 5, 447-458.

VARGAS, E. A., SANTOS, E. A., PITTET, A. (2002) Collaborative Study submitted for consideration by *AOAC International*: D-2 Protocol - Determination of ochratoxin A in green coffee by immunoaffinity column clean up and LC. Ministry of Agriculture, Livestock and Supply, LACQSA/LAV-MG, Belo Horizonte, Brasil.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