

**FIRST RESEARCH COORDINATION MEETING (RCM) OF THE FAO/IAEA
COORDINATED RESEARCH PROGRAMME (CRP) ON "EVALUATION OF
METHODS OF ANALYSIS FOR DETERMINING MYCOTOXIN
CONTAMINATION OF FOOD AND FEED"**

KUALA LUMPUR, MALAYSIA, 26-30 APRIL, 1999

BACKGROUND

The present **Co-ordinated Research Programme** is to complement the FAO/IAEA Training and Reference Centre (TRC) for Food and Pesticide Control under the Centre's mission "to assist Member States and their institutions to fulfil requirements to support the implementation of international standards/agreements relevant to food safety and control, the safe use of pesticides and sanitary and phytosanitary measures, by providing training, quality assurance services and technology transfer."

Based on the Global Environment Monitoring System - Food Contamination Monitoring and Assessment Programme (GEMS/Food) data and other national data on mycotoxin contamination, mycotoxins are a widespread problem of the food supplies in most countries. As a result, many countries have enacted regulations to control the level of mycotoxins in the national food supply as well as in food moving in international trade. At the international level, the Codex Alimentarius Commission, through its Committee on Food Additives and Contaminants and relevant commodity committees, is considering the establishment of international guideline levels for various mycotoxins based on risk assessments performed by the Joint FAO/WHO Expert Committee on Food Additives. Codex activities are of particular importance in view of the World Trade Organization (WTO) Sanitary and Phytosanitary Measures (SPS) Agreement as well as on the WTO's Agreement on Technical Barriers to Trade. The SPS Agreement specifically refers to Codex standards, guidelines and recommendations as representing the international consensus on health and safety requirements for food based on sound scientific risk assessment. This will require national authorities to give greater attention to the development of consistent and standardised approaches to regulations and their enforcement, including sampling and methods of analysis.

Consequently, it is essential that the analytical capabilities of laboratories in developing countries are strengthened in order to enable them to effectively monitor the mycotoxin content of food in trade to overcome the non-tariff barriers based on the Agreement on Application of Sanitary and Phytosanitary Measures of the World Trade Organization.

There is a need to develop research data on the effectiveness of the various analytical methods, including radioimmunoassays, used by food control laboratories to monitor mycotoxin contamination in order to select and recommend cost-effective validated procedures.

OBJECTIVES

The overall objective of this CRP is to assist national food control authorities and institutions to improve food safety and stimulate international trade in food by identifying and validating time and cost efficient methods for detection and quantification of mycotoxins in food in order to effectively monitor the mycotoxin content of agricultural import and exports.

The specific objective of this RCM is to review the programme of work to be carried out by the participants under the scope of the CRP. The RCM will among others:

- *Identify high risk mycotoxin/commodity combinations that present impediments to trade through regulations and/or are non-tariff barriers to trade
- *Evaluate analytical methods used by food control laboratories in developing and advanced countries for detection and quantification of these mycotoxins
- *Select time and cost efficient validated methods best suited for analyzing the high risk mycotoxin/commodity chosen which may be adaptable to developing country use.
- *Make recommendations to developing countries of an established portfolio of appropriate and harmonized analytical methods with good performance characteristics.

A second objective of the CRP is to support the activities of the FAO/AEA Training and Reference Centre for Food and Pesticide Control of the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture under its mandate "to assist Member States and their institutions to fulfil requirements to support the implementation of international standards/agreements relevant to food safety and control, the safe use of pesticides and sanitary and phytosanitary measures by providing training, quality assurance services and technology transfer.

The benefits of the CRP are expected to include:

- *Standardised analytical methods adapted to developing country use
 - *Validated procedures
 - *Publications including TECDOC and manuals
 - *More effective use of laboratory resources: time and money
 - *Improved implementation and compliance with legislation
- *Submission of harmonized methods for endorsement by AOAC and Codex Methods Committee

THE MEETING

The meeting was hosted by the Malaysian Institute for Nuclear Technology Research (MINT), and held at the Federal Hotel in Kuala Lumpur, 26-30 April, 1999. It was attended by all contract and research holders from seventeen institutions from the following fifteen countries: Argentina, Australia, Brazil, Canada, China, Cuba, Egypt, Indonesia, Italy, Malaysia, Philippines, South Africa, United Kingdom, Uruguay, and USA.

The meeting was opened by the Director General of MINT Y Bhg. Dato' Dr. Ahmad Sobri Haji Hashim, by Mr. Ezzeddine Boutrif of the Food Quality and Standards Service of FAO, and by the Scientific Secretary of the meeting Dr. Maya Pineiro of the Food and Environmental Protection Section, Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture. The list of participants of the RCM is attached as Annex I.

The objective of the meeting was to examine the scope and objectives of the CRP and agree on aspects of common interest to CRP participants.

To attain this the following activities were undertaken during the meeting:

1. An introduction address on Global Trends in Mycotoxin Control and Analytical Methods; a presentation of the Joint FAO/IAEA Division's Program as related to mycotoxins and definition, procedures and working aspects of CRP's were presented by the scientific secretary.
2. Research proposals were presented by each contract and agreement holder. Copies of

the presentations are included as Annex II.

3. The initial objectives of the CRP were discussed and confirmed as originally stated with emphasis on the applicability of methods in developing countries. These were:

a) select suitable mycotoxin/commodities combinations (high risk and those most likely to be affected by regulations)

b)select analytical methods for experimental trials

c)agree on procedures for validation, collaborative study and interlaboratory tests

d) review work plans of participants of the CRP for the next 5 years

4. The proposals presented by the participants were considered and evaluated in the context of the whole CRP.

5. Suitable mycotoxin/commodities combinations and analytical methods for experimental trials were selected.

6. Proposed methodology and implementation programme for each selected mycotoxin/commodities combination and method were discussed extensively and agreed upon.

It was decided that instead of individual research carried out by each participant as originally proposed in the contracts and agreements, workgroups would be defined to study jointly a selected method/mycotoxin/commodities combination with defined tasks and areas of coordination and cooperation between researchers.

7. Validation/Performance Criteria were set for all collaborative and ring trials according to the International Harmonized Protocol: A minimum of 8 data sets with 3 levels of naturally contaminated materials, as blind duplicates and two pairs of blanks (one pair to be spiked) would be used. The Horrat ratio should be less than 2.

The following seven workgroups were created:

Workgroup 1: Aflatoxin in maize and peanut butter

Participating contract/agreement holders: UK (NRI), China, Egypt, Indonesia, Cuba, Uruguay, Argentina, UK (MAFF), USA. for initial development trials and all CRP participants for collaborative trials.

Coordinator: UK (NRI/MAFF)

Workgroup 2: Fumonisin B1 in maize

Participating contract/agreement holders : Egypt, Indonesia, Cuba, Malaysia, Australia, China, UK (NRI), Philippines, Uruguay, USA, Canada (Scott), South Africa, Argentina, Italy.

Coordinator: South Africa

Workgroup 3: Aflatoxin M1 in milk

Participating contract/agreement holders: Argentina, UK (MAFF), Uruguay, Brazil, Philippines, Malaysia, China.

Coordinator: Argentina

Workgroup 4: Ochratoxin A in coffee

Participating contract/agreement holders: Brazil, Italy, Canada (Scott), USA.

Coordinator: Brazil

Workgroup 5: Feasibility study of ELISA technology for mycotoxins in developing countries

Participating TC, contract/agreement holders: Australia, China, Indonesia, Egypt, Cuba.

Coordinator: Australia

Workgroup 6: Preparation of radiolabelled fumonisin for disposition studies in naturally contaminated corn

Participating TC contract/agreement holders: Canada (Miller), Italy.

Coordinator: Canada (Miller)

Workgroup 7: Multiple trichothecenes in wheat and corn

Participating contract/agreement holders: Argentina, Italy, USA, UK (MAFF), South Africa, Cuba, Egypt, Brazil, Uruguay.

Coordinator: Uruguay

A complete timeframe for the whole CRP for the next two years including the tasks for each contract/agreement holder was approved (Annex III).

The specific objectives, workplans, outline, timeframe and tasks for each workgroup and each participant were decided, approved and assigned as follows:

WORKPLAN OUTLINES AND TIMEFRAMES

WORKGROUP 1: DEVELOPMENT AND VALIDATION OF LOW-COST, ROBUST, EASY TO PERFORM METHOD FOR AFLATOXINS IN FOODS AND FEEDS APPROPRIATE FOR USE IN DEVELOPING COUNTRIES

This project addresses the question of whether new developments in clean-up techniques which are generally employed with HPLC end-determination can in fact be successfully adapted for use with TLC. The project will address the question of

robustness of any proposed method and will uniquely carry out a full validation study with participants from developing countries.

OBJECTIVES:

- 1.To assess the viability of adapting an existing SPE/HPLC/HPTLC method for aflatoxins to be applied with a visual TLC end-point.
- 2.To establish the practicality and conditions for multiple use of affinity columns for sample clean-up of aflatoxins from foodstuffs.
- 3.To establish the availability and ruggedness of affinity columns under conditions pertaining in developing countries.
- 4.Subject to successful outcomes to 2 and 3 to assess the viability of adapting an existing affinity column method for aflatoxins to be applied with a visual TLC end-point.
- 5.To assess the outcomes of 1 and 4 and decide whether to pursue full collaborative trial evaluation of either SPE/TLC or affinity column TLC methods.

SCOPE:

Commodities to be covered:- maize and peanut butter.

Performance to be achieved:-to work to a limit of 2 ng/g aflatoxin B₁

SPE column:- phenyl bonded column

ISSUES:

Whether to still pursue trial if it proves not possible to meet 2 ng/g limit of detection i.e. to pursue trial but with materials containing higher levels of aflatoxins.

Whether alternatively to pursue trial with densitometry or HPLC if it proves impossible to meet 2 ng/g limit of detection by TLC.

TASKS TO BE UNDERTAKEN:

Task 1

NRI (UK) + China

Adaptation of SPE methodology to a TLC end-point.

May to October 1999

Task 1.1

Egypt + Indonesia + Cuba

Test robustness of proposed SPE/TLC method

June to October 1999

Task 2

Uruguay + Argentina

Establish conditions for multiple use of affinity columns

May to October 1999

Task 3 UK (MAFF + NRI) + Uruguay
Decision on whether to pursue SPE/TLC or affinity column/TLC approach
October 1999

Task 4 UK (MAFF)
Distribute maize practice samples + standards
October 1999

Task 4.1 All trial participants (12 +)
Undertake aflatoxin B1 analysis using spikes and report results
October 1999 to Feb. 2000

Task 5 MAFF (UK)
Draft protocol for full collaborative trial for approval by AOAC International
October 1999 to Feb 2000

Task 6 USA
Preparation of maize test material at three levels of contamination plus blank material – test material to be ground to small particle size, mixed and packaged in 100g test portions.
May 1999 to October 1999

Task 7 USA
Establishment of homogeneity of test materials –(demonstrate no difference between inter-unit CV and intra-unit CV – analysis of every 10th sample from batch)
May 1999 to October 1999

Task 8 UK (MAFF)
Distribution of samples, standards and collaborative trial protocol
March 2000

Task 9 All (10+ partners)
Undertake collaborative trial
March 2000 to June 2000

Task 10 MAFF (UK + NRI)
Collation of trial results, statistical analysis and drafting of publication.
June 2000 to August 2000

Task 11 All partners
Test robustness of method locally by analysing survey samples.

WORKGROUP 2 COLLABORATIVE STUDY OF TLC METHOD FOR DETERMINING FUMONISIN B1 IN MAIZE

OBJECTIVE

1. To establish a sensitive TLC method for fumonisin B1 in maize
2. To collaboratively study the proposed method according to AOAC International guidelines

WORKING GROUP

Amra
Bahri
Garcia
Hamid
Kennedy (Vietnam)
Liu
Nagler (help)
Padilla
Pineiro
Pohland (help)
Scott (help)
Shephard (Coordinator)
Sola
Visconti (help)

OUTLINE OF METHOD

The method to be studied represents the use of appropriate technology, instruments and solvents in order to make the final product viable for use in laboratories in developing countries. The outline of the method to be developed and studied is as follows:

- 1. Extraction.** Maize samples will be extracted by shaking for 1 hour using methanol/water (3:1). Although it is recognised that acetonitrile/water (1:1) and blending may produce marginally improved extraction efficiencies, these were considered to be inappropriate in the laboratories in which this method will be ultimately used.
- 2. Clean-up.** Sample clean-up will be performed by SAX SPE cartridges. The use of

reversed-phase C18 cartridges will not produce an extract suitable for TLC determination at low levels.

3. Quantification. Quantification will be by TLC. This is regarded as essential for the routine use of the method in laboratories in developing countries, but presents the project with the need to undertake initial developmental work.

INITIAL DEVELOPMENT REQUIREMENTS

(Subgroup: Shephard, Scott, Visconti, Nagler)

This subgroup will undertake initial development of the TLC method. The currently used TLC method utilises reversed-phase C18 plates and fluorecamine as a spray reagent. A pre-concentration zone allows a relatively large volume to be loaded. Its limit of detection is thought to be realistically around 200 ng/g. A method published by M. Nagler that uses normal phase plates developed by dipping in *p*-anisaldehyde solution is suitable for rice, but produces significant interferences with maize samples.

The subgroup will investigate the use of spray or dip reagents with C18 and normal phase TLC and HPTLC plates.

TIME FRAME AND WORK PLAN

The fumonisin standards and SAX cartridges needed will be supplied to participants in the study.

May-Oct 1999: 1. Investigate TLC spray reagent (Shephard, Scott, Visconti, Nagler)
2. Acquire maize (Pohland, Shephard)
3. Approach additional labs (Potential extra participants from India, Ghana, South Africa)(Shephard, Pineiro)

Nov-Dec 1999: Communicate TLC results to participants. They can test the system in their own labs (Shephard, volunteers)

Nov-Jan 2000: Prepare AOAC International submission (Shephard)

Feb-Apr 2000: 1. First round of study to give participants lab experience of the new method. It will involve the provision of standards, maize samples and sample extracts in order to test the various stages of the method. (all participants)
2. Prepare maize samples and test homogeneity. (Pohland)

May 2000: Assess first round results (All participants)

June-Sep 2000: Second round of study. Full collaborative study on naturally

contaminated maize using blind duplicates of 4 naturally contaminated maize samples (including a blank) and a recovery on a known blank in which participants spike the samples with an unknown concentration. (All participants)

Oct-Dec 2000: 1. Assess results (All participants)
2. Consider further action (All participants)
3. Write report (Shephard)

WORKGROUP 3 DETERMINATION OF METHODS FOR AFLATOXIN M1 IN MILK.

OBJECTIVE:

Select and Validate a Method for Aflatoxin M1 in Milk.

SELECTED METHOD:

Immunoaffinity Column Method

Following the IDF Standard 171: 1995 in procedure but changing the Detection Step to TLC instead of HPLC.

-Immunoaffinity Column performance check

PROJECT WORK PLAN:

<u>Task1</u>	MAFF (UK)	Completed by June 1999
Procure materials for collaborative trial		
Aflatoxin M1 standard to be procured from CNEVA (France)		
Raw milk naturally contaminated at 2 ng/mL to be obtained from CNEVA for dilution to target levels of contamination for collaborative trial purposes.		
<u>Task 2</u>	LATU (Uruguay)	Completed by July 1999
Draft protocol for collaborative trial		
<u>Task 3</u>	INTI (Argentina)	Completed by Sept. 1999
Establish affinity column re-use conditions		
Column to be tested to establish the maximum number of times it can be re-used before there is a reduction in recovery of aflatoxin M1 and optimum conditions for re-use to be established.		
<u>Task 4</u>	INTI (Argentina)	Completed by Sept. 1999
Establish conditions for affinity column clean-up combined with TLC		
<u>Task 5</u>	INTI (Argentina)	Completed by Nov. 1999
Distribute samples and protocol to participants		
<u>Task 6</u>	INTI (Argentina)	Completed by Jan. 2000
Compile sample results .		

WORKGROUP 4: OTA IN COFFEE

OBJECTIVE

SELECT ANALYTICAL METHOD FOR OTA IN COFFEE

Evaluate and validate method

Nestle / Brazil
EC / Dr Visconti

Working Group

Sabino , Visconti, Scott, FDA (Trucksess)

Start: June / 99

The preliminary test will be develop in Institute Adolfo Lutz

Workplan

1. Establish the chromatographic conditions and test cleanup (Immunoafinity column).
IAL June 99
2. Test material will be obtained from producer, green coffee.
3. Test material will be preliminary tested in green coffee from Nestle August-October 99
4. Test material will be tested in green coffee by October 99
5. Establish conditions for ring stuy, organize with ITAL, UNICAMP, UNESP, by 12/99
6. Draft protocol
7. Perform ring trial, June 2000
8. Compile results
9. Submit results as AOAC extension as additional matrix for AOAC official method, August 2000.

WORKGROUP 5: ELISA FOR MYCOTOXINS

Participants

1. Dr Liu Xiumei

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3. Professor Ivan Robert Kennedy

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Title

Feasibility study on the application of ELISA technology to the analysis of mycotoxins in developing countries

Activity

The working group will investigate the role of the ELISA technique, using specific antibodies to mycotoxins with enzyme linked immunosorbent assay. Immunoassays for aflatoxin B1 (polyclonal, Bahri and Kennedy) and fumonisins B1 (monoclonal) are under development. Dr. Bahri is preparing an ELISA kit with a view to using it for purposes of field testing to screen agricultural produce, using laboratory analysis to confirm contamination of positive samples. Dr. Liu intends to apply the ELISA test kit to a study of human populations in China with high and low rates of occurrence of oesophageal cancer. before this can be done, further work to provide a reliable ELISA kit will be necessary. the kit prepared at the University of Sydney will be employed in Vietnam's Post-Harvest Technology Institute (Dr. Le Van To, Director) in Ho Chi Minh city for a baseline field survey of aflatoxins in agricultural produce (maize and groundnuts) in Vietnam. This survey will be conducted as part of a collaborative project funded by the Australian Center for Agricultural Research (ACIAR, 1999-2002) – "Monitoring Mycotoxins in Grain and Food Production Systems for Risk Management", seeking to apply ELISA

technology for a range of mycotoxins.

The activities at these three centres will be coordinated by I.R. Kennedy from the University of Sydney, with a view to sharing information, overcoming problems and providing quality assurance for the tests to be developed. The group will maintain contact via a 3-monthly tele-conference prepared by email/fax, with progress to be reported to Dr Maya Pineiro by I.R. Kennedy.

Specific issues to be addressed include:

The need to have appropriate ELISA kits available at reasonable cost. The solution adopted at these three centres is to develop expertise for antibody production and prepare kits from locally-produced materials in the context of local cost structures. The ELISA kits developed by the working group will be bench-marked when possible against commercially available kits. A kit prepared in Cuba available at more reasonable cost (report of Dr. Miguel Garcia, icidca@ceniai.inf.cu) may be included in this benchmarking if available and found suitable.

Methodology for laboratory analyses. Issues to be examined and experiences shared relate to the problems of sampling and extraction, suitable solvents, overcoming matrix effects, suitable sensitivity and specificity, robustness and ease of use. Where opportunities arise, kits can be interchanged between the laboratories for performance testing.

Methodology for field testing. Formats for field testing (e.g. tube tests with reagents added by dropper bottles, more advanced lateral flow display of positives, etc.) with the advantages of appropriate sensitivity, ease of use and reliability will be sought.

Validation of the results of field surveys using laboratory methods. A common protocol will be sought.

The ELISA Working Group will jointly review the literature for appropriate advances and report on its progress in its objectives, with recommendations, to the next meeting of the CRP.

WORKGROUP 6: TECHNIQUES FOR EVALUATING DISPOSITION OF FUMONISINS IN NATURALLY-CONTAMINATED CORN DURING FOOD PROCESSING

J. David Miller, Carleton University, Ottawa
Angelo Visconti, Institute of Toxins and Mycotoxins, CNR, Bari

OBJECTIVES

To develop a method to produce corn kernels infested with *Fusarium verticillioides* and contaminated by fumonisin produced in vivo enriched by C14 at sufficient activity to permit the material to be ground into meal and used to evaluate the disposition of fumonisins in food products made from corn meal in comparison to data from chemical analysis.

RATIONALE & METHOD

There are many data that demonstrate that the analysis of processed food containing fumonisin B1-contaminated corn meal or corn flours is not comparable to the analysis of raw grain corn. Thermal processing (baking, extrusion, autoclaving, frying), the presence of specific compounds in corn that bind fumonisin B1, as well as the addition of other ingredients to the food that may chelate (iron) hydrolyze (base) or chemically bind to fumonisin B1 (sugars), fumonisin and fumonisin fragments may be changed or may not be extracted by existing methods of chemical analyses

One solution to this would be to add 14C -labeled fumonisin. Such materials are available; it is unambiguously known that C1-C2 14C acetate applied at the correct time in controlled liquid culture fermentations produce evenly labeled fumonisins. The difficulty is that spiking corn with purified FB1 is not the same as the form that fumonisins might be present in vivo in corn kernels. The alternative is to produce corn kernels containing fumonisin from incubation in vitro and enriched by 14C. It is known that the by addition of 14C acetate to naturally infected corn kernels, the in vivo growth rate can be estimated by the determination of incorporation of 14C into ergosterol. It is also unambiguously known that 14C fumonisin can be produced by the incubation of infested corn kernels and adding an appropriate radiolabelled precursor.

It should therefore be possible to produce corn kernels containing fumonisin enriched with 14C. There are a number of serious technical barriers particularly how to avoid the 14C going primarily into ergosterol, how to remove unused 14C precursor and to achieve reasonable levels of radioactivity in the fumonisins.

OUTPUT IN 1999-00

The two labs will work together to solve the methodological problems with a goal to determining how to produce the desired materials in 1999-00. In subsequent years, materials will be produced and used to undertake pilot experiments in processed corn foods on the fate and disposition of naturally contaminated fumonisins. Cooperations will have to be developed with labs in countries that consume products not made in North America or western Europe, such as with labs in South America and Asia. These materials may also be used for recovery studies in the analytical program.

WORKGROUP 7: METHOD FOR MULTIPLE TRICHOHECENES

OBJECTIVE

Select, evaluate and validate multiple trichothecenes method for wheat and corn

SELECTED METHOD

AOAC/EC with home made columns and TLC application.

WORKING GROUP

Sola, Visconti, Pohland (Mary Trucksess), Gilbert, Shepard, Garcia, Amra, Sabino, Pineiro (coordinator)

TIME FRAME

Will start on the second year of the CRP, 4/2000. The task will be kept in deferred mode until the EC-SMT Program's DON project has reached a more advanced stage.

Extraction recommendations and method modification will be adapted accordingly

PROJECT WORKPLAN

Task 1-The AOAC / EC method will be preliminary tested and EC recommendations incorporated. Establish conditions for collaborative trial.

Task 2- Standards will be obtained and provided to participants

Task 3- Test materials will be obtained from the EC program or from FDA's sources.

Task 4- Draft protocol.

Task 5- Distribute samples + protocol

Task 6- Compile results