行政院所屬各機關因公出國人員出國報告書

(出國類別：研究實習)

真菌毒素之分子生物檢驗技術

服務機關：衛生署藥物食品檢驗局

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內容摘要: 爲能提升食品中真菌毒素之檢驗技術與品質及瞭解美國對真菌毒素之管理及監控方式，藥物食品檢驗局（以下簡稱爲本局）派員赴美國食品藥物管理署（U.S. Food & Drug Administration,）CFSAN（Center for Food Safety and Applied Nutrition）進行檢驗技術之交流。此研習之目的爲（1）與負責專家討論真菌毒素污染食品之管理與監控方法（2）真菌毒素檢驗之技術交流（3）建立本局與CFSAN之交流與合作。本次研習與CFSAN真菌毒素專家Dr. Mary W. Truckness等人研討有關於美國真菌毒素之限量標準、檢驗方法、管理及監控方法等相關議題，並實際操作黃麴毒素、伏馬毒素、赭麴毒素、棒麴毒素、Deoxynivalenol及T-2 toxin等真菌毒素之檢驗。對於食品中真菌毒素之行政管理及檢檢驗技術層面都獲益良多，相信對於未來食品中真菌毒素之預防以及處理有實質之助益。

本文電子檔已上傳至出國報告資訊網

真菌毒素之分子生物檢驗技術

摘　要

為能提升食品中真菌毒素之檢驗技術與品質及瞭解美國對真菌毒素之管理及監控方式，藥物食品檢驗局（以下簡稱為本局）派員赴美國食品藥物管理署(U.S. Food & Drug Administration,)CFSAN(Center for Food Safety and Applied Nutrition）進行檢驗技術之交流。此研習之目的為（1）與負責專家討論真菌毒素污染食品之管理與監控方法（2）真菌毒素檢驗之技術交流（3）建立本局與 CFSAN 之交流與合作。

本次研習與CFSAN真菌毒素專家Dr. Mary W. Truckness等人研討有關於美國真菌毒素之限量標準、檢驗方法、管理及監控方法等相關議題，並實際操作黃麴毒素、伏馬毒素、赭麴毒素、棒麴毒素、Deoxynivalenol 及 T-2 toxin 等真菌毒素之檢驗。對於食品中真菌毒素之行政管理及檢檢驗技術層面都獲益良多，相信對於未來食品中真菌毒素之預防以及處理有實質之助益。

目次

前言與目的

臺灣位於亞熱帶之島嶼，濕熱之天氣易使儲存之食品受真菌污染而產生真菌毒素危害人體健康，且隨著台灣加入 WTO，國際間商品之區域性間隔逐漸消失，一些於國內以往少見之食品中真菌毒素可能出現於國內之消費市場。

為維護國人之飲食安全，此行考察美國食品藥物管理署（U.S. Food & Drug Administration,；以下簡稱為 FDA）之 CFSAN（Center for Food Safety and Applied Nutrition；以下簡稱為 CFSAN）之目的為（1）與負責專家討論真菌毒素污染食品之管控方法（2）真菌毒素檢驗之技術交流（3）建立本局與 CFSAN 之交流與合作。相信對於未來食品中真菌毒素之預防以及處理有實質之助益。

內　容

此行於 CFSAN 研習之主要單位為 Division of Natural Products，行程共計 14 日，扣除往返行程及例假日，實際於 CFSAN 研習之日數為 9 天。研習內容包涵 FDA 對進口與國產食品中真菌毒素之監控、真菌毒素之限量標準及檢驗方法、收集真菌毒素之相關訊息等，並與 FDA 之研究人員實際操作真菌毒素之檢驗方法。研習內容整理後摘要如下所述：

（一）美國食品中真菌毒素之限量標準、檢驗之方法及方法之擬訂：

拜會 Bioanalytical Chemistry Branch 之 Branch Chief Dr. Mary W.

Truckness，討論真菌毒素之限量標準及分析方法（附件一及二）。由於 Dr. Truckness 兼任 AOAC 真菌毒素分析方法之評審員，並曾多次舉辦真菌毒素分析方法之 collaboratory study，故請教 Dr. Truckness 方法建立及審核等相關議題（附件三及四）。

（二）美國國產及進口之食品中真菌毒素管控方針：

拜會 Compliance Program Branch 之 Branch Chief Mr. Ronald R. Roy。經由與 Mr. Roy 討論得知，FDA 對於美國國產或進口食品之真菌毒素管控主要針對 Aflatoxins（黃麴毒素）、Patulin（棒麴毒素）、Deoxynivalenol（以下簡稱為 DON）、Fumonisins（伏馬毒素）、Ochratoxin A（赭麴毒素），並依據 FDA 制訂之 Compliance Program Guidance Manual（附件五）進行抽樣及分析。

（三）食品中真菌之鑑定及真菌之毒素萃取及毒性檢驗：

與 Mycologist Dr. Valerie Tournas 學習從食品中可疑的產毒真菌，利用培養後之萃取液是否造成 brine shrimp 死亡鑑定是否為產毒之真菌（附件六）。

（四）黃麴毒素之檢驗：

與 Research Chemist Mr. Michael E. Stack 學習利用三氟醋酸管柱前衍生化反應（pre-column）與利用新儀器 PHRED（Photochemical Reactor For

Enhanced Detection）進行管柱後衍生化反應（post-column），所得層析結果黃麴毒素 B1、B2、G1 及 G2 之 peak 出現順序差異，做為檢驗結果之確認方法（附件七）。

（五）伏馬毒素之檢驗：

與 Mr. Stack 學習玉米中伏馬毒素之檢驗（附件八）。此方法之特點於使用有機溶液（乙腈：水：醋酸＝500：500：10）取代 Mr. Stack 先前於 AOAC 發表以鹽類溶液（甲醇：0.1M 磷酸二氫鈉＝77：23）為層析時之移動相（鹽類易結晶而影響層析結果）。

（六）黃麴毒素及赭麴毒素之檢驗：

由於黃麴毒素及赭麴毒素均可由 *Aspergillus* 屬之真菌所產生，目前 Dr. Truckness 及 Mr. Stack 正研究如何同時萃取及分析兩種真菌毒素，但由於因不同食品基質所造成毒素萃取純化後干擾因子差異大，故在此僅截錄利用梯度層析（gradient）同時分析黃麴毒素及赭麴毒素之標準品（附件九）。

（七）棒麴毒素之檢驗：

與 Mr. Stack 學習蘋果汁中棒麴毒素之檢驗（附件十）。此方法以紫外光偵側器判讀結果與本局使用之螢光偵側器為主要差異點。

（八）DON 之檢驗：

與 Dr. Robert Eppley 學習玉米 DON 之檢驗（附件十一）。

（九）T-2 toxin 之檢驗：

與 Dr. Truckness 學習利用 ELISA 檢驗 T-2 toxin（附件十二）。

心　得

美國從 911 攻擊事件後，對反恐怖主義工作不餘遺力，而食品更被美國民眾公認為最易受恐怖攻擊且最不易防範的項目，故 CFSAN 成為反恐重點機關之一，每次進入大門時宛如登機檢察，須通過警衛金屬探測器檢察，手邊行李也須經 X 光照射檢察。而 CFSAN 中，許多研究計劃與反恐怖相關，此類研究之內容與結果均被列為機密並未對外公開。Dr. Truckness 之部門主要職責進行方法之建立、限量標準之擬定訂、突發案件處理、並與 AOAC 合作進行新檢驗方法之 collaboratory study 及審合，而一般市售產品及進口商品則交由 FDA 之 district lab 依據 Compliance Program Guidance Manual 進行審核、調查及監控。另經 Dr. Truckness 介紹有幸拜會 Division of Science and Applied Technology 之 Director Dr. George Perry Hopkin 討論海洋毒素之議題，但很遺憾由於多數海洋毒素如河鈍毒素及痲痺性貝毒等屬急毒性，已被列為反恐實驗，故無法實際參與操作相關議題之實驗。

建　議

1.此次美國 CFSAN 之行承蒙周家璜博士鼎力協助得以成行，在此特別表示感謝。周博士建議本局應陸續派員造訪 CFSAN 之不同部門以建利良好之合作關係，且 CFSAN 內之華裔人士已屆臨退休之年紀，未來恐無法繼續協助介紹與連絡之工作。

2.國內食品衛生標準目前僅對食品中黃麴毒素有限量標準之規定，本局於兩年前始針對與食品安全有關之重要真菌毒素陸續進行方法之建立及污染背景值之調查，將有助於未來對食品中各種真菌毒素之管控。進而建立類似CFSAN制訂之Compliance Program Guidance Manual供國內食品中真菌毒素監控之基準。

3.Dr. Truckness 之實驗室有一位協助實驗之工讀生為美馬里蘭州大之學生以減少人員經費支出並可與學術單位建立合作關係，建議未來本局未來可增加在學生以替代助理。

4.赴國外考查、研習等雖然花費高，但可經由面對面之技術交流建立良好友誼，且在此電子通訊發達的年代，回國後仍可以繼續交流與合作。故建議往後能與其他不同之先進實驗室間進行交流。

附　件

附件一

Mycotoxins of Health Concern

Mary W. Trucksess, Ph.D.
U.S. Food and Drug Administration
Center for Food Safety and Applied Nutrition

Mycotoxins

- Secondary metabolites produced by certain fungi during growth in the field, processing, transport or storage
- Natural occurrence and unavoidable
- Toxic and/or carcinogenic
- Low molecular weight, most <1000 dalton
- Vary in stability to processing

Efforts to Control Mycotoxins

- Setting regulatory limits or guidelines
- Monitoring of susceptible products
- Implementing decontamination procedures
- Diverting contaminated grains to industrial uses

Current Worldwide Regulations for Mycotoxins

- More than 80 countries have taken steps to regulate mycotoxins, in particular the aflatoxins.
- Regulations vary depending upon whether the country setting the limit is an importer or exporter.
- Many have followed the US lead.
- Many others have taken the approach that, when the mycotoxin is a carcinogen, no amount is acceptable.

Major Mycotoxins

- Aflatoxins
- Fumonisins B_1, B_2 and B_3
- Deoxynivalenol
- Ochratoxin A
- Patulin

Compliance Programs for Mycotoxins in Foods

- Compliance Program Guidance Manual
- Chapter 07 - Molecular Biology and Natural Toxins

Implementation

Objectives: FY-2002

- Collect and analyze food products for aflatoxins, fumonisins, deoxynivalenol (DON), ochratoxin A and patulin
- Remove (domestic) or detain (imported) foods containing aflatoxins and patulin at levels above regulatory guidelines

Collecting Sample

- Typical sample size is 3-20 kg
- Large particle sizes require a larger sampling size
- Juice 12 units
- US Department of Agriculture recommends:
  - Corn, milo, wheat 5-10 lb
  - Peanuts, Brazil nuts 48 lb

Sample Collection

Mycotoxin	Domestic Samples	Imported Samples
Aflatoxins	800	500
Fumonisins	400	200
DON	125	80
Ochratoxin A	125	80
Patulin	400	200

Special surveillance and compliance samples

Aflatoxins

- Produced by *Aspergillus flavus* and *A. parasiticus*
- Found in corn, cottonseed, peanuts, tree nuts copra, and milk
- Bioactivated aflatoxin B_1 can bind to DNA, RNA and proteins
- IARC has classified as a probable human carcinogen

U.S. FDA Compliance Policy Guides for Aflatoxins (ng/g)

All food products except milk	20
Corn or peanuts for immature animals and dairy cattle	20
Corn or peanuts for breeding beef, cattle, swine, and mature poultry	100
Corn or peanuts for finishing swine	200
Cottonseed meal (feed ingredient)	300
All feedstuff other than corn, peanuts	20
Milk (aflatoxin M_1)	0.5

Sample Collections for Aflatoxins

- Domestic: corn, corn meal or flour, grits, snack food; peanut (roasted), peanut butter, other nuts; pumpkin seeds.
- Imported: same as domestic; in addition collect: pine nuts, sunflower seeds, melon seeds, ginger.

Analytical Methods for Aflatoxins

AOAC Official Method of Analysis, Chapter 49, 16th edition, 1995.

- AOAC 991.31 (immunoaffinity column cleanup, LC separation and quantitation).
- AOAC 994.08 (Mycosep column cleanup, LC separation).
- AOAC 968.22 (CB method, silica gel column cleanup, TLC separation).

Analytical Methods for Aflatoxins

Confirmation of identity procedures for regulatory samples:

- AOAC 975.37, chemical derivatization method (peanuts, corn, pistachio nuts, pumpkin seeds, cottonseed meal).
- AOAC 985.17, mass spectrometric method.

Fumonisins

- Produced by *Fusarium moniliforme* and *F. proliferatum*
- Found in corn, rice, and sorghum
- Fumonisin contaminated corn may show no visible evidence of mold damage
- Cause leukoencephalomalacia in horses, pulmonary edema in swines, liver cancer in rats, and kidney cancer in mice

American Association of Veterinary Laboratory Diagnosticians Guidance Levels for Fumonisin B_1 in Feed

Animal	Feed	Level
Horse	Non-roughage portion of diet	5ppm
Swine	Total ration	10ppm
Poultry	Total ration	50ppm
Beer cattle	Non-roughage portion of ration	50ppm
Dairy cattle	No recommendation	

Fumonisin - Regulatory Guidelines

- No regulations or action level issued by FDA
- Document to provide guidance to industry for levels adequate to protect human and animal health was published in the Federal Register, June 6, 2000
- Pending

Fumonisin Guidance Document

- Guidance levels

  Human exposure to fumonisins should not exceed levels achievable through good agriculture and manufacturing practices
- Recommended levels are presented in the document

Fumonisin Guidance Levels for Human Foods

Product	Total Fumonisins ($B_1 + B_2 + B_3$) ppm
Degermed dry milled corn products	2
Dry milled corn bran	4
Cleaned corn intended for masa production	4
Cleaned corn intended for popcorn	3

Fumonisin Guidance Levels for Animal Feeds

Corn and corn by-product intended for	Total Fumonisins ($B_1 + B_2 + B_3$) ppm
Equids and rabbits	5 (< or = 20% of diet)[1]
Swine and catfish	20 (< or = 50% of diet)[1]
Breeding ruminants, breeding poultry & breeding mink[2]	30 (< or = 50% of diet)[1]

[1]Dry weight basis

[2]Includes lactating dairy cattle and hens laying eggs for human consumption

Fumonisin Guidance Levels for Animal Feeds

Corn and corn by-product intended for	Total Fumonisins ($B_1 + B_2 + B_3$) ppm
Ruminants ≥ 3 months old for slaughter and mink being raised for pelt production	60 (< or = 50% of diet)[1]
Poultry being raised for	100 (< or = 50% of diet)[1]
All other species or classes of livestock and pet animals	10 (< or = 50% of diet)[1]

[1]Dry weight basis

Sample Collections for Fumonisins

- Domestic: Milled corn (flaking grits, corn bran, corn grits, corn germ), breakfast cereal (ready to eat, quick cooking and instant), infant and Junior cereals, corn muffin (dry mix), corn meal, sorghum syrup.
- Imported: Same as domestic.

Analytical Methods for Fumonisins

- AOAC 995.15, SAX column cleanup, OPA derivatization, LC separation and quantitation.
- Immunoaffinity column cleanup, LC separation and quantitation
- Enzyme-linked immunosorbent assay (ELISA)
- C_{18} SPE cleanup, OPA derivatization, LC separation and quantitation.

Deoxynivalenol (DON, Vomitoxin)

- A 12,13-epoxytrichothecene
- Produced by *F. graminearum* and other F. spp.
- Found on cereal crops: wheat, corn, barley.
- Known to cause feed refusal, emesis, and growth depression in swine.
- Associated with acute gastrointestinal illness in humans (China and India).

U.S. FDA Advisory Levels for Deoxynivalenol

- ⌘Bran, flour, and germ for human consumption — 1ppm
- ⌘Grain and grain by-products for beef cattle and feedlot cattle and for chickens — 10ppm
- ⌘Grain and grain by-products for swine and all other animals — 5ppm

Sample Collections for Deoxynivalenol

Deoxynivalenol

- ⌘Domestic: Whole wheat flour, white flour, and wheat bran.
- ⌘Imported: wheat flour, wheat bran

Analytical Methods for Deoxynivalenol

AOAC Peer Verified Method II (PVM 2), J.AOACI 1998.

Extraction: acetonitrile-water

Cleanup: Romer MycoSep column 225

LC separation and quantitation: C18, 15% methanol, 220 nm

Ochratoxins

- ⌘Produced by *Aspergillus ochraceus* and related species and *Penicillium* spp.
- ⌘Found in corn, barley, wheat, oats, and green coffee beans
- ⌘Ochratoxin A causes kidney damage in animals, Ochratoxin A may be the cause for Balkan kidney disease
- ⌘IARC has determined ochratoxin A as an animal carcinogen

Ochratoxin A - Regulatory Guidelines

- ⌘No regulations or guidelines issued by FDA
- ⌘FDA supports the use of good agricultural practices and good manufacturing practices (GMPs) to keep levels of ochratoxin A to the lowest level feasible.
- ⌘CCFAC is preparing Code of Practice for reducing ochratoxin A levels in cereal grains.

Sample Collection for Ochratoxin A

Barley (whole), barley malt, baby cereals, cereals (corn, oat, rice, wheat, barley), corn meal, dried beans/peas, coffee beans, raisin, rye flour, soya based baby food products, wheat flour, oats

Analytical Methods for Ochratoxin A

- AOAC 991.44, silica gel SPE, LC separation and quantitation.
- Immunoaffinity column cleanup, LC separation and quantitation

Patulin

- Produced by *Pencillium expansum*
- Found in rotten apples, pears, and cherries
- Mutagenic, neurotoxic, immunotoxic, genotoxic, and causes gastrointestinal effects in rodents
- No formal evidence of carcinogenic risk

U.S. FDA Compliance Policy Guides for Patulin (ng/mL, pending)

Apple juice and apple products	50

Sample Collections for Patulin

Apple juice, apple juice concentrate.

Method for Patulin AOAC Method 995.10

- Patulin is extracted with ethyl acetate and then cleaned up by extraction with sodium carbonate solution.
- Extracted sample is dried with anhydrous sodium sulfate. Ethyl acetate is evaporated.
- Patulin is determined by reversed-phase LC with UV detection

Future Work on Mycotoxins

- International harmonization of regulatory levels, sampling, methods
- Laboratory certification
- Quality assurance
- Reference materials, check samples

附件二

Liquid Chromatograhic Methods for Mycotoxins

Mary W. Trucksess, Ph.D.
Food and Drug Administration
Center for Food Safety and Applied Nutrition
Division of Natural Products

Reasons for the Need of Methods

- ⌘Compliance with legislation
- ⌘Monitoring and survey work
- ⌘Control at stages of food and feed production
- ⌘Research: toxicological study, decontamination, epidemiology study
- ⌘Risk assessments

Methods of Analysis

- ⌘Quantitative or qualitative
- ⌘Specific for one mycotoxin
- ⌘Specific for multimycotoxins
- ⌘Confirmatory

Analytical Techniques for Mycotoxins

- ⌘Thin layer chromatography (TLC)
- ⌘Liquid chromatography (LC)
- ⌘Gas chromatography (GC)
- ⌘LC/Mass spectrometry (MS)
- ⌘GC/MS
- ⌘Immunoassay
- ⌘Fluorometry
- ⌘Biosensor

Critical Factors for LC Separation

- ⌘Sampling
- ⌘Extraction
- ⌘Purification
- ⌘Concentration
- ⌘Equipment
- ⌘Reagents
- ⌘Detection technique

Liquid Chromatographic Methods

- ⌘LC column:
  - normal phase (not common)
  - reversed phase
  - ion exchange
- ⌘Injector: manual, or automatic
- ⌘Pump: single, or multisolvent delivery
- ⌘Detector: UV, fluorescence
- ⌘Post-column derivatization reactor

Liquid Chromatographic (LC) Methods for Major Mycotoxins

- ⌘Aflatoxins
- ⌘Fumonisins
- ⌘Deoxynivalenol
- ⌘Ochratoxin A
- ⌘Patulin

Aflatoxins

- ⌘Produced by *Aspergillus flavus* and *A. parasiticus*
- ⌘Found in corn, cottonseed, peanuts, tree nuts copra, and milk
- ⌘Bioactivated aflatoxin B_1 can bind to DNA, RNA and proteins
- ⌘IARC has classified as a probable human carcinogen

LC Methods for Aflatoxins Extraction & Cleanup

- ⌘Extraction solvent: water-saturated chloroform, aqueous methanol, acetonitrile, acetone
- ⌘Cleanup: silica gel column, immunoaffinity column, C18, solid phase extraction

LC Methods for Aflatoxins Conditions

- ⌘LC condition: isocratic reversed-phase
- ⌘Mobile phase: H_2O + CH_3OH + CH_3CN
- ⌘Pre-column derivatization: TFA
- ⌘Post-column derivatizaton: aqueous iodine, cyclodextrins, pyridinium bromide perbromide, problem: an additional pump, KOBRA cell (electrochemical cell), PHRED cell (photochemical reactor)
- ⌘Detection: Ex 360 nm Em 420 nm

Automated LC Methods for Aflatoxins

- ⌘ On line immunoaffinity column (one-time use) then C18 SEP clean up followed by reversed phase LC for aflatoxin M_1 in milk
- ⌘ On line immunoaffinity column (>50 time use) cleanup, LC separation, KOBRA cell bromine derivatization, for aflatoxins in corn and peanuts
- ⌘ Robotic system for sample cleanup and LC separation and detection for M1 in milk

AOAC Official LC Methods for Aflatoxins

- ⌘ Method 990.33 2g silica gel SPE column clean up (solvent efficient method), TFA derivatization, for corn and peanut butter
- ⌘ Method 991.31 immunoaffinity column (IAC) cleanup, post column iodine derivatization, for corn, raw peanuts and peanut butter
- ⌘ Method 999.07 IAC cleanup, post column bromine, for peanut butter, pistachio nuts, figs and paprika
- ⌘ Method 2000.08 IAC cleanup, for M1 in milk

Fumonisins

- Produced by *Fusarium moniliforme* and *F. proliferatum*
- Found in corn, rice, and sorghum
- Fumonisin contaminated corn may show no visible evidence of mold damage
- Cause leukoencephalomalacia in horses, pulmonary edema in swines, liver cancer in rats, and kidney cancer in mice

LC Methods for Fumonisins Extraction and Cleanup

- Extraction solvent:
  - Methanol + H_2O (3 + 1)
  - Acetonitrile + H_2O (1 + 1)
  - Acetonitrile + methanol + H_2O (25 +25 +50)
  - Acetonitrile + pH3 buffer (1 + 1)
- Cleanup: solid phase extraction (SPE), strong anion exchange (SAX), C18, immunoaffinity column

LC Methods for Fumonisins Derivatization

- The fumonisins no chromophore UV absorption, require derivatization to form fluorescent compound
- Derivatization reagents & derivative stability
- o-phthaldialdehyde (OPA) + 2-mercapto-ethanol + borate buffer, pH 9-10, unstable
- Naphthalene-2,3-dicarboxaldehyde (NDA) + KCN, stable

LC Methods for Fumonisins Conditions

- Column: C18, Ultracarb (Phenomenex), μBondapak (Waters), AQ column (YMC)
- Mobile phase:
  - Acetonitrile-water-acetic acid (50 + 50 + 1), elution order FB1, FB2, FB3
  - Methanol + 0.1M phosphate buffer pH 3.35 (77 + 23), elution order FB1, FB3, FB2
- Detection: Pre-column derivatization, Ex 335 nm, Em 440 nm

Deoxynivalenol (DON, Vomitoxin)

- Produced by *F. graminearum* and other spp.
- *F. graminearum* causes head blight in wheat and ear rot in corn
- Found in corn, barley, rye, wheat
- Inhibitor of protein and DNA synthesis

LC Method for Deoxynivalenol

- Extraction solvent: CH_3CN-H_2O (84 + 16)
  - Extraction:
  - 3 min blending for spiked feed
  - 16 min blending for natural contaminated feed
- Cleanup: Solid phase extraction (SPE), a mixture of charcoal, alumina, and Celite or MycoSep 225 column

LC Method for Deoxynivalenol

- Mode: isocratic, or step gradient
- Column: C18, 5 μM, 4.6 mm x 10 cm, Spheris (Perkin Elmer)
- Mobile phase:
  - Water-acetonitrile-methanol (92 + 4 + 4)
  - Water-methanol (85 + 15) then (1 + 1)
- Detection: UV 220 nm

Ochratoxin A

- Produced by *Aspergillus ochraceus* and related species and *Penicillium* spp.
- Found in corn, barley, wheat, oats, and green coffee beans
- Ochratoxin A causes kidney damage in animals, Ochratoxin A may be the cause for Balkan kidney disease
- IARC has determined ochratoxin A as an animal carcinogen

LC Methods for Ochratoxin A

- Extraction solvent:
  - 0.1 M phosphoric acid + chloroform (1+10)
  - Methanol + 1% sodium bicarbonate (1+1)
- Cleanup: Solid phase extraction, C18, immunoaffinity column
- LC conditions: reversed phase, C18 column, fluorescence detection, Ex 333 nm, Em 460 nm

Common Problems with LC

- Mechanical problems: leaky fittings, leaks at pump, injector leaks, column leaks, detector leaks
- Mobile phase problems: solvent purity, insufficient degassing, wrong composition
- Chromatogram problem: peak tailing, interfering peak, split peak, extra peaks, broad peaks retention time drift, baseline noise

References for Mycotoxin LC Methods

- Trucksess, M.W., Pohland, A.E. (eds.) (2000) Methods in Molecular biology, Vol. 157, Mycotoxin Protocols, Humana Press, Totowa, New Jersey, 244 pp.
- Trucksess, M.W. (Chapter Editor), (2000) Chapter 49, Natural Toxins, In: Official Method of Analysis, 17th Edition, W. Horwitz, ed., AOAC International, Gaithersburg, MD

LC Methods for Mycotoxins References

- Miraglia M., Brera, C. (2000) Determination of mycotoxins in grains and related products. In: *Food Analysis by HPLC*, 2nd edition L.M.L. Nollet ed., Marcel Dekker, NY, pp. 493-522
- Phenomenex, HPLC Trouble-Shooting, Torrance, CA
- Scott, P.M., Trucksess, M.W. (1997) Application of immunoaffinity columns to mycotoxin analysis, J. AOAC Int. 80, 941-49

LC Methods for Patulin References

- Brause, A., Trucksess, M.W., Thomas, F.S., Page, S.W. "Determination of patulin in apple juice: collaborative study", J. AOAC Int. 79, 451-455 (1996).
- Trucksess, M.W. and Tang Y. (1999) "Solid-phase extraction method for patulin in apple juice and unfiltered apple juice" J. AOACI, 82, 1109-1113.

LC Methods for Patulin References

- Roach, J.A.G., White, K.D., Trucksess, M.W., and Thomas, F.S. (2000) "Capillary gas chromatography/mass spectrometry with chemical ionization and negative ion detection for confirmation of identity of patulin in apple juice." J. AOACI, 83, 104-112.

References

- AOAC International, (2000) *Quality Assurance Principles for Analytical Laboratories*, 3rd Edition, AOAC International, Gaithersburg, MD
- AOAC International, (2000) *ISO 17025 and the Laboratory--An Introduction to Laboratory Accreditation*, AOAC International, Gaithersburg, MD

附件三

Mycotoxin Method Evaluation

Mary W. Trucksess, Ph.D.
Food and Drug Administration
Center for Food Safety and Applied Nutrition
Division of Natural Products

Mycotoxins in Foods and Feeds

Uncertainty
Incidence and Levels

Mycotoxins in Foods and Feeds

- Reasons for Uncertainty
- Sampling large quantities of foods
- Performance of analytical methods
- Evaluation of data quality

Analytical Methods

- Sampling
- Sample preparation
- Extraction
- Purification
- Isolation
- Determination
- Confirmation

Sampling Plan

Sampling plan describes the sequence of activities that provides a laboratory sample which can be analyzed for the selection of mycotoxins

Sampling Sequence

- Defining the aim of sampling
- Identifying a sampling point
- Identifying a lot
- Selecting a sampling method
- Collecting a sample
- Performing sample preparation
- Producing a laboratory sample

Collecting Sample

- Typical sample size is 3-20 kg
- Large particle sizes require a larger sampling size
- US Department of Agriculture recommends:

Corn, milo, wheat	5-10 lb
Peanuts	48 lb
Brazil nuts	48 lb

Sample Preparation

- Sample is ground in a mill to reduce particle size
- A subsample is removed from the comminuted subsample for extraction

Analytical Procedure

- Extraction: water + organic solvent
- Purification: Solid phase extraction, liquid-liquid partition, precipitation (chemical, or dilution).
- Quantitation: Immunoassay (IA), Chromatographic methods: thin layer (TLC), liquid (LC), Gas (GC), GC/Mass spectrometric (MS), LC/MS, Capillary zone electrophoresis (CZE).
- Confirmation of Identity: Chemical Derivatization, MS analysis

Method of Analysis

- Selection of appropriate method
- Use of authentic toxin standards of known purity
- Validation of the method for use with a particular commodity.

Factors in Choosing a Method

- Mycotoxins of interest
- Sample matrix, sample preparation
- Dynamic range, detection limit
- Location of analysis (grain elevators, processing facilities, analytical labs)
- Training of the analysts
- Ease of use, cost

Criteria for Selection of a Method

- Experience required of the analyst
- Cost of equipment
- Number of analyses
- Time required
- Location of analysis
- Waste disposal
- Safety

Sources of Mycotoxin Standards

- Commercial sources: Sigma Chemical, Supbelco
- Private sources: researchers
- Isolation from fungal cultures

Calibration and Purity Tests of Standards

- Melting point
- Visible/ultraviolet spectroscopy
- Nuclear magnetic resonance
- Infrared spectroscopy
- Mass spectrometry
- Chromatography

Method Evaluation

Determine performance characteristics of the method:

Selectivity and specificity
Range, linearity, sensitivity
Limit of detection
Limit of quantitation
Accuracy, and precision.

Three AOAC Method Validation Programs

- Official Methods℠ (OMA)
- Performance Tested Methods℠ (PTM)
- Peer-Verified Methods℠ (PVM)

Minimum Validation Requirements for OMA

Quantitative Methods

- 5 materials (combination of matrix and levels for a single analyte)
- 8 laboratories
- Blind duplicates or Youden pairs

Minimum Validation Requirements for OMA

Qualitative Methods

- 5 materials, 2 levels for each material, and
  5 negative controls
- 15 laboratories
- Blind duplicates or Youden pairs

AOAC ® Peer-Verified Methods℠ *(PVMs)*

- Requirement:
  Originating laboratory plus a second, independent laboratory

AOAC ® Performance Tested Methods℠ *(PTMs)*

Requirement:

Third party verification of performance claims for commercial proprietary test kits/analytical according to AOAC Research Institute (RI) protocol.

Analyst Method Validation

- Conduct recovery studies for each matrix at levels of toxicological concern.
- Compare results (accuracy and precision, limit of detection, limit of determination) of the study with data of the collaborative studies of published methods.

Obtaining Credible Data

- Use validated method in the laboratory
- Implement laboratory quality assurance program
- Follow standard operating procedures
- Maintain sample integrity and traceability
- Use reference material, standard, control chart
- Participate proficiency testing

The Use of Credible Data

- For obtaining science-based risk assessments
- For setting regulatory limits
- For managing mycotoxins in food and feed
- For controlling mycotoxins
- For ensuring safety of food supply

附件四

AOAC Method Validation and Analytical Methods for Mycotoxins

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Food and Drug Administration
Center for Food Safety and Applied Nutrition
Division of Natural Products

Major Mycotoxins in Food

Toxin	Carcinogenicity Human	Animal	IARC Classification
Aflatoxins	S	S	1
Aflatoxin M_1	I	S	2B
Ochratoxin A	I	S	2B
Zearalenone		L	
Fumonisin B_1		L	
Deoxynivalenol		I	
Patulin	I	I	3B

S = sufficient, I = inadequate, L = limited evidence
1 = carcinogenic to humans, 3 = not classifiable
2B = possibly carcinogenic to humans

Strategic Plan for the Control of Mycotoxins

Science-based risk analysis

- Hazard identification: bioassays, isolation and characterization of mycotoxins, epidemiological studies.
- Risk assessment: analytical methods, toxicological studies, compliance programs, risk assessment models
- Risk management: regulatory limits, monitoring, processing, HACCP

Mycotoxin Analytical Method Separation and quantitation

- Thin layer chromatographic methods
- Liquid chromatographic methods
- Gas chromatographic methods
- Mass spectrometric methods
- Immunochemical methods
- Other novel methods

Mycotoxin Analytical Methods Confirmation of Identities

- Chemical derivatization
- Mass spectrometric analysis

AOAC Validation of a Method

Establishes, through systematic laboratory studies, that the performance characteristics of the method meet the specifications related to the intended use of the analytical results.

AOAC Validation of a Method

Performance characteristics determined include selectivity and specificity, range, linearity, sensitivity, limit of detection, limit of quantitation, accuracy, and precision.

Why Are Validated Methods Important to the Laboratory?

- Promote common regulatory and industry standards
- Support laboratory accreditation
- Contribute to sound methods used worldwide
- Entry to additional standards organizations like CODEX, ISO, CIPAC, IDF

Why Are Validated Methods Important to the Laboratory?

- Improve employee development and experience in scientific review, organization, management, and communication
- Strengthen confidence in the validity of methods used in your laboratory

Three AOAC Method Validation Programs

–Official Methods℠ (OMA)

–Performance Tested Methods℠ (PTM)

–Peer-Verified Methods℠ (PVM)

People Involved in Inter-Laboratory Collaborative Study

- Collaborators
- Methods Program Manager
- Associate Referee
- General Referee
- Committee Safety Advisor
- Committee Statistics Advisor
- Methods Committee Members
- Official Methods Board Members
- AOAC Members (Mail Ballot)
- AOAC Staff

Use of Official AOAC Methods

- Regulatory Analysis
- Required in the Enforcement of Food & Drug Law
- In Research to Judge a Method's Reliability Using the Performance Data
- For Quality Control Purposes
- In Contract Specifications

AOAC ® Official Methods℠ *(OMA)*

OBJECTIVE:

To provide analytical methods for which performance characteristics have been validated to the highest degree of confidence -- through independent, multi-laboratory collaborative study.

Minimum Validation Requirements for OMA

QUANTITATIVE METHODS

- 5 materials (combination of matrix and levels for a single analyte)
- 8 laboratories
- Blind duplicates or Youden pairs

Minimum Validation Requirements for OMA

QUALITATIVE METHODS

- 5 materials, 2 levels for each material, and
  5 negative controls
- 15 laboratories
- Blind duplicates or Youden pairs

Status and Publication for OMA

- Adopted as AOAC® Official Method℠, first action
- Published in the compendium, Official Methods of Analysis
- Collaborative study report published in the Journal of AOAC INTERNATIONAL, or another scientific journal
- After 2 years of use, method may be recommended for adoption final action

Revisions or Modifications to OMA

- Modifications:
  - Editorial
  - Minor change
  - Method extension (applicability statement, matrix)
  - Analyte addition
  - Procedure change
  - Substantive revision

Revisions or Modifications to OMA

- Amount of supporting information, data, or study is dependent on extent of modification
- All modifications go through the same levels of review as methods recommended for adoption

OMA TLC and HPLC Methods Individual Mycotoxins

- Aflatoxins
- Deoxynivalenone
- Ochratoxins
- Patulin

AOAC Official Methods (IAC) for Mycotoxins

Mycotoxin	Manufacturer	Commodity	ng/g
Aflatoxins	Vicam	corn peanuts	10
Aflatoxins	Neogen	almonds peanuts	5[1,2]
Aflatoxins	Vicam	peanut butter, pistachios, figs, paprika	2
Aflatoxin M_1		milk	0.02[1]
Fumonisins	Vicam	wine, feed[1]	
Ochratoxin		roasted coffee	1.2[1]
Ochratoxin		barley	1.3[1]

[1]Collaborative studies completed.
[2]Reusable for 5 times

AOAC Official Methods (ELISA) for Mycotoxins

Mycotoxin	Manufacturer	Commodity	ng/g
Aflatoxin B_1 (B_1)	Neogen (Agri-screen)	corn, peanuts mixed feed	20
B_1, B_2, G_1	Int. Diag. Sys. (Cup test)	Corn, peanuts peanut butter, cottonseed	20
B_1, B_2, G_1, G_2	Biokits	peanut butter	9
Fumonisins	Neogen	corn, feed	1000
Zearalenone	Neogen	corn, wheat, feed	800

AOAC ® Peer-Verified Methods℠ *(PVMs)*

- **Objective:**
  To provide a class of validated methods for currently developed techniques where rapid validation must occur in about 12 months' time
- **Requirement:**
  Originating laboratory plus a second, independent laboratory

Performance Parameters for PVMs

Quantitative Methods

- Specificity, Linearity
- Limit of Detection
- Limit of Quantitation
- Precision (Repeatability)
- Comparison to Reference Method
- Ruggedness, Matrix, Recovery
- Limited Reproducibility

Status and Publication of PVM

- Accepted as AOAC ® *Peer-Verified Methods*℠
- Published as individual methods
- Verification study is published in *JAOAC*
- Methods reviewed every 5 years

Revisions and Modifications of PVMs

- May be modified as needed by users
- Modifications, with supporting data may be submitted to AOAC and published as "Notes" attached to original method

AOAC Peer-Verified Method for Mycotoxins

AOACI PVM 2

- Deoxynivalenol (DON) in white flour, whole wheat flour, and bran.
- Romer MycoSep column 225 cleanup, reverse phase HPLC separation, UV detection at 220 nm
- DON > 0.5 µg/g

AOAC ® Performance Tested Methods℠ *(PTMs)*

- Objective:
  To provide 3rd party verification of performance claims for commercial proprietary test kits/analytical
- Methods Requirement:
  Verification of performance claims according to AOAC Research Institute (RI) protocol

Performance Parameters for PTMs

Quantitative Methods

- Specificity, Linearity
- Limit of Detection
- Limit of Quantitation
- Precision (Repeatability)
- Comparison to Reference Method
- Ruggedness, Matrix, Recovery
- Limited Reproducibility
- Package Insert Review
- Quality Policy Certification

Performance Parameters for PTMs

Qualitative Methods

- Specificity rate, Sensitivity rate
- False Positive and False Negative Rates
- Percent Method Agreement
- Ruggedness, Matrix, Recovery
- Limited Reproducibility
- Package Insert Review
- Quality Policy Certification

Status and Publication for PTMs

- Certified as Performance Tested Method℠
- Method validation report published in *JAOAC*

Revisions and Modifications for PTMs

- Any changes to kit require notification to AOAC RI
- Approved test kits must be renewed annually to maintain certification status

AOAC Research Institute Certification Mark

PERFORMANCE TESTED
AOAC
RESEARCH INSTITUTE
LICENSE NUMBER 960902

AOAC Performance Tested Methods for Mycotoxins

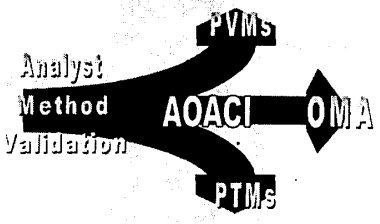
Mycotoxin	Test Kit	Manufacture
Aflatoxins	Aflatest P	Vicam
Aflatoxins	Veratox AST	Neogen
DON	Veratox for DON	Neogen
DON	Ridascreen Fast	r-Biopharm
T-2 Toxin	Ridascreen Fast	r-Biopharm

Analyst Method Validation

- Choose AOACI validated method or published method.
- Conduct recovery studies for matrix of interest at levels of toxicological concern.
- Compare results (accuracy and precision, limit of detection, limit of determination) of the study with data of the AOAC method or published method.

The Use of Credible Data

- For obtaining science-based risk assessments
- For setting regulatory limits
- For managing mycotoxins in food and feed
- For controlling mycotoxins
- For ensuring safety of food supply



附件五

FOOD AND DRUG ADMINISTRATION
COMPLIANCE PROGRAM GUIDANCE MANUAL

PROGRAM	7307.001

CHAPTER 07 - MOLECULAR BIOLOGY AND NATURAL TOXINS

SUBJECT:	IMPLEMENTATION DATE
MYCOTOXINS IN DOMESTIC FOODS FY 02/03/04	October 1, 2001
	COMPLETION DATE September 30, 2004

DATA REPORTING

PRODUCT CODES	PRODUCT/ASSIGNMENT CODES
See Attachment A	07001 All sample collections and analysis

FIELD REPORTS TO HEADQUARTERS

1. Report all collections/analyses of domestic **food** samples for aflatoxin, patulin, deoxynivalenol, fumonisin, or ochratoxin A* contamination against this program even though the samples were collected during inspections scheduled under other compliance programs.

2. The analyzing district will report analytical results into FACTS using the PAF = "MYC" and insure that the correct Mycotoxin Code **for the mycotoxins analyzed for** is used.

3. * When entering information into FACTS, use operation code 31 to report domestic sample collection and operation code 41 to report domestic sample analysis. *

PART I - BACKGROUND

A. General

* Mycotoxins are toxic metabolites produced by certain fungi that can infect and proliferate on various agricultural commodities in the field and/or during storage. The occurrence of these toxins on grains, nuts and other commodities susceptible to mold infestation is influenced by environmental factors such as temperature, humidity, and extent of rainfall during the pre-harvesting, harvesting, and post-harvesting periods. Mycotoxins may exhibit various toxicological manifestations; some are teratogenic, mutagenic and /or carcinogenic in susceptible animal species and are associated with various diseases in domestic animals, livestock, and humans in many parts of the world.

The occurrence of mycotoxins in foods and feeds is not entirely avoidable; therefore small amounts of these toxins may be in foods and feeds. Under section 402(a)1 of the Federal Food, Drug and Cosmetic Act, a food is deemed adulterated if it contains poisonous or deleterious substance, such as mycotoxins, which may render it injurious to health. Mycotoxins can be considered added poisonous or deleterious substances because their presence in human food and/or animal feeds can be avoided in part by good agronomic and manufacturing practices. Strategies used by the Food and Drug Administration (FDA) to minimize mycotoxins in the U.S. food supply include establishing guidelines (e.g., action levels, guidance levels), monitoring the food supply, through formal compliance programs (domestic and import) and **taking regulatory action against product that exceeds action levels, where action levels have been established.** The monitoring data obtained over the years from FDA's monitoring programs is used to provide: (a) estimates of the incidence and levels of contamination by various mycotoxins in affected areas in the country, (b) dietary exposure data (estimates) for use in making risk assessments for specific mycotoxins, (c) background data for use in considering the establishment of guidelines for specific mycotoxins, (d) an estimate of the economic impact of the enforcement of regulatory guidelines on foods and feeds during a given crop year, (e) information needed to prepare answers to governmental questions including congressional inquiries, and (f) basic information needed to support the position and recommendations of U.S. delegates participating in international meetings. The monitoring data also serves as a database describing the background distribution of various mycotoxins in domestic grains and their products in the U.S. as a function of geographic area and environmental conditions.

B. Specific Mycotoxins to be included in this program

1. **Aflatoxins**, metabolic products of the molds *Aspergillus flavus* and *A. parasiticus*, may occur in food as a result of mold growth in a number of susceptible commodities, including peanuts and corn. Other domestic nuts and grains are susceptible but less prone to contamination with aflatoxins. Because aflatoxins are known carcinogens to laboratory animals, and presumably to man, the presence of aflatoxins in foods should be restricted to the [illegible] practical levels attainable using modern processing techniques. The current action level for total aflatoxins in [illegible] level for aflatoxin M_1 in milk is 0.5 micrograms per kilogram (0.5

ppb).

2. **Patulin** is a toxic substance produced by *Penicillium, Aspergillus,* and *Byssochylamys* molds that may grow on apples. Since Patulin is not destroyed by heat processing, and can occur at high levels in apple juice, including pasteurized apple juice, if rotten, moldy or damaged apples are used to make juice, both pasteurized and non-pasteurized single strength juice and concentrated juices are to be collected. Animal feeding studies have demonstrated that high levels of patulin in apple juice could pose a health risk if the juice is consumed over an extended period of time. In 2001, FDA established an action level of 50 micrograms per kilogram (50 ppb) for patulin in apple juice and in the apple juice component of a food that contains apple juice as an ingredient. The action level is based upon the patulin level in single strength apple juice, reconstituted single strength apple juice (if the food is an apple juice concentrate), or the single strength apple juice component of the food (if the food contains apple juice as an ingredient). Single strength juice is 100 percent juice that is unconcentrated (see 21 CFR 101.30(h)). Under the juice Hazard Analysis Critical Control Point (HACCP) regulations set to take effect from 2002-2004, some apple juice processors may need to establish control measures such as using only tree picked fruit, and culling their apples to be used for juice production to remove rotten and damaged fruit.

3. **Deoxynivalenol (DON)**, commonly called vomitoxin is a natural toxin produced by several molds of the genus *Fusarium,* especially *F. graminearum,* which is a common contaminant of several grains, including wheat, corn, barley, and rye. DON has been associated with a number of adverse health effects in humans and animals. Several adverse weather related DON contamination episodes in the U.S. have motivated the FDA to issue guidance levels for food (wheat) and feed in 1982 and updated levels in 1993. (See Part VI, Additional References, #7.) FDA is continuing to study the scope and toxicological significance of the DON problem to determine if further regulatory measures are needed to control DON in food and feed products. The FDA has established a guidance level of 1 microgram per gram (1 ppm) for deoxynivalenol in finished wheat products that may be consumed by humans. No guidance level has been established for raw wheat intended for milling into human food products.

4. **Fumonisins** (Fumonisin FB_1 and Fumonisin FB_2) are natural toxins produced by *Fusarium verticillioides* (previously known as *F. moniliforme),* and other *Fusarium* species; these molds are common natural contaminants of corn. Fumonisins have been linked to fatalities in horses and swine. Recent studies have demonstrated the presence of fumonisins in human foods, including corn meal and breakfast cereals. Epidemiological investigations demonstrating a possible association of *F. verticillioides* with esophageal cancer and recent animal studies indicating the carcinogenicity of fumonisin FB_1 have highlighted the need to ensure that foods do not contain excessive amounts of fumonisins. Dry milling of whole corn kernel generally results in the production of fractions called bran, flaking grits, grits, meal, and flour. Because fumonisins are concentrated in the germ and the hull of the whole corn kernel, dry milling results in fractions with different concentrations of fumonisins. For example, dry milled fractions

(except for the bran fraction) obtained from degermed corn contain lower levels of fumonisins than dry milled fractions obtained from non-degermed or partially-degermed corn. Industry information indicates that dry milling results in fumonisin-containing fractions in descending order of highest to lowest fumonisin levels: bran, flour, meal, grits, and flaking grits.

The FDA has established the following guidance levels for fumonisins ($FB_1+FB_2+FB_3$) in foods.

Guidance Levels for Fumonisins in Foods	Total Fumonisins ($FB_1+FB_2+FB_3$)
Degermed dry milled corn product	2 ppm
Whole/partly degermed dry milled corn product	4 ppm
Dry milled corn bran	4 ppm
Cleaned corn intended for popcorn	3 ppm
Cleaned corn for masa production	4 ppm

Note: These levels are more commonly referred to as micrograms per gram but because limitations in electronic transmission cause the symbol for microgram to be distorted or omitted, the ppm unit of measurement is being used.

5. **Ochratoxin A** is a naturally occurring nephrotoxic fungal metabolite produced by certain species of the genera *Aspergillus* and *Penicillium*. It is mainly a contaminant of cereals (corn, barley, wheat and oats), and has been found in edible animal tissues as well as in human blood sera and milk. Studies indicate that this toxin is carcinogenic in mice and rats. It is not completely destroyed during the processing and cooking of food, therefore the implication of risk to human health and safety must be considered. FDA needs current up-to-date information on the incidence and levels of occurrence of this toxin in the U.S. for use in considering any necessary regulatory control measures for this substance. The recommended analytical method that will be used has a lower limit of quantitation than methods employed in previous field assignments.

C. **Products that are to be sampled include:**

1. ***Apple Juice and Apple Juice Concentrate**

Apple juice and apple juice concentrate are to be collected for patulin analysis. If samples are collected in conjunction with the inspection of an apple juice processor, consider collecting samples if the processor does not cull apples to be used to produce juice (including stored apples) to remove rotten apples and visibly damaged apples i.e. bruising, breaks in the skin, holes, visible mold, hail damage, bird pecks. Consider collecting samples also if it is established that the processor uses drops i.e., apples that have fallen from the tree and are harvested from the ground, (also known as grounders, windfalls or ground fruit) to produce juice. *

2. **Corn and Corn Products**

Historically, aflatoxin levels in corn have been highest in the Southeastern states. Corn from anywhere in the U.S. may be affected, however, depending on the growth, harvesting and storage conditions involved, as was the case in the Mid-west in 1988 and in Texas in 1987.

Aflatoxin levels, in food products made from corn (grits, meal, flour, snack foods or cereals), are likely to be higher in "full fat" than in degerminated products, since the highest levels of aflatoxin in the kernel are associated with the germ.

FDA samples of fresh, frozen and/or canned sweet corn have been analyzed for the presence of aflatoxin and no violative samples found in the past. Therefore, this program does not call for sampling of sweet corn for aflatoxin analysis.

*Samples of shelled corn (designated for human use), corn meal, corn based snacks, and corn based breakfast cereals (corn flakes, grits) will be collected and analyzed for the presence of aflatoxins.

The corn meal and corn based cereal products listed above will also be collected and analyzed for ochratoxin A.

Samples of whole, partially degermed, and degermed dry-milled corn products (flour, meal, grits, flaking grits, bran) and cleaned corn intended for masa production and for popcorn (unpopped) will be collected and analyzed for the presence of fumonisins.

3. **Milk**

When dairy animals consume feed containing high levels of aflatoxin, one of the metabolized aflatoxins (aflatoxin B_1) may be secreted into the animals' milk as aflatoxin M_1. Cattle consuming feed that contains less than 20 ppb of total aflatoxins, however, should produce milk that complies with FDA's guideline of 0.5 ppb for aflatoxin M_1 in milk.

4. **Peanuts**

Raw: * Testing of raw peanuts, domestic and imports, for aflatoxins is conducted by USDA in accordance with the FDA/USDA MOU (See Sec. 570.375 CPG 7112.02). *

Processed: The testing for aflatoxin in roasted in-shell and shelled peanuts, as well as processed peanut products for consumer use, is the responsibility of FDA. In general, the varieties, grade, and geographical growing area for peanuts used for roasting have resulted in low aflatoxin levels in roasted-in-shell peanuts. However, when there are shortages of the usual varieties used for roasting, a variety of "Runner" peanut grown in northern Florida may be substituted. Peanuts of this variety and from this area have consistently had a relatively high incidence and level of aflatoxin contamination.

Other Nuts

Almonds, macadamia pecans, pistachios, and walnuts are susceptible to aflatoxin contamination, but samples of these domestic nuts have been largely in compliance (less than 1% adverse) for several years. FDA surveillance of these crops, however, is necessary to assure that industry-implemented quality control procedures continue to effectively prevent the marketing of aflatoxin contaminated nuts.

6. **Wheat Products**

Milled wheat products (whole wheat flour, white flour and bran) will be collected at wheat product manufacturers. Some samples of bran that may be used as a component of bran cereal, but not the cereal itself, may be collected at cereal manufacturers. All of these samples will be analyzed for deoxynivalenol.

7. **Other Products**

Rye flour, wheat flour, barley (cereals), oats (whole/cereals) dried beans, soya flour and soya based baby foods will be collected and analyzed for ochratoxin A.

PART II - IMPLEMENTATION

OBJECTIVES

To collect and analyze domestic samples of various food products to determine the occurrence and levels of aflatoxins, patulin, fumonisins, deoxynivalenol, and ochratoxin A;

To remove from interstate commerce those foods that contain aflatoxins *or patulin* at levels judged to be of regulatory significance;

*To provide a database to support FDA's positions in international activities concerning mycotoxins. *

PROGRAM MANAGEMENT INSTRUCTIONS

Federal/State Relations

State officials are valuable sources of information on current and potential aflatoxin problems in foods. In the past, a number of states have participated in an aflatoxin data exchange program with FDA. Districts should encourage state participation in this data exchange program and should coordinate aflatoxin program activities with State officials to prevent duplication of efforts in both food and feed sampling. Information on this data exchange program can be obtained from the Division of Federal-State Relations, HFC-150, (303) 443-3360.

PART III - INSPECTIONAL

A. Inspectional

This program does not direct inspections. However, time is allocated for follow-up inspections if violative levels of aflatoxin *or patulin* are detected in regulatory (not *surveillance*) samples.

Inspectional guidance for mycotoxin inspections is contained in Section 8 of DEIO Guide to Inspections of Manufactures of Miscellaneous Food Products Volume II.

B. Sampling

General Guidance:

*Refer to the current ORA workplan and to Attachment A of this compliance program for all sample obligations, i.e., number of samples for each mycotoxin to be collected and the products for each mycotoxin to be collected.

It is imperative that the products specified in Attachment A, for each analyte, be collected and analyzed as a unique sample; therefore, a sample from a single lot of a product is **not** to be collected for multiple mycotoxin analyses. Specifically, a product collected for aflatoxin, fumonisin or ochratoxin analysis is not to be analyzed for more than one mycotoxin. In the past, with CFSAN's concurrence, samples were bundled, i.e., a single sample was collected for multiple mycotoxin analysis. This approach did not end up yielding the requested analyte/product combinations. *

Mycotoxin contamination can occur in localized pockets at high concentrations in foods such as unprocessed grains and nuts. For sampling bulk products, representative samples should be obtained by using a trier or other device that will provide representative portions from all sections of the container sampled. Commodities such as fruit juice, other fluid items, and mixed preparations (paste, spreads, butters) are generally homogenous and do not require any special devices for sampling.

If not referenced below, additional sample sizes are referenced in the Investigations Operations Manual (IOM), Chapter 4, Sample Schedule Chart 6. Collect samples randomly so as to be representative of the lot. Sample only the foods listed in Attachment A. Except for patulin analysis and except for the number of surveillance subsamples and size of surveillance subsamples (as listed in sections 3, 4 and 5 below) the information in Sample Schedule Chart 6 should be followed.

1. **Aflatoxin**

a. * Sample only the foods listed in Attachment A, Section 1.

If the District wishes to sample another product, **BEFORE** sampling consult the Domestic Mycotoxin Monitor, Robyn Jones, at (301) 436-2575. If she cannot be reached and an immediate answer is needed, contact Dr. Garnett Wood at (301) 436-1942, and notify Robyn Jones by e-mail.

Various corn-based foods such as tacos, chips, cereals and snack foods are acceptable for sampling under this program *for aflatoxin testing only*. However, it is preferable to sample the corn ingredient that will be used in manufacturing these products.

Consider using ultra-violet light (blacklighting) as a screening technique, prior to sampling shelled corn, if many lots are available for sampling at one location. In some cases, corn contaminated by molds will fluoresce a bright yellowish green color. See IOM 427.4g; Blacklight Test Screening Procedure for Aflatoxins in Corn, for guidance on procedure.

b. Do not collect more than 2 samples of any specific commodity at any firm, unless there is a need to collect more samples of that commodity for compliance purposes.

c. Do not sample in-shell peanuts (except the "Runner" variety of peanut when roasted in-shell) or nut meats destined for processing that is intended to remove aflatoxin contaminated nuts.

d. Do not sample raw peanuts except as a follow-up to violative levels of aflatoxins in a finished product or when raw peanuts are offered directly to consumers. This is in accordance with the FDA/USDA MOU. (Sec. 570.375 CPG 7112.02).

e. Do not sample popcorn unless there is reason to believe that aflatoxin contamination may be present due to late harvest or adverse environmental conditions. The characteristics of the cultivar of corn used for popping make it unlikely to be aflatoxin contaminated.

f. Sample milk for aflatoxin M_1 if state coverage is inadequate in areas where the potential for aflatoxin in dairy rations exists. Use the results of the District's sampling of feed under Center for Vet. Medicine directives and the results of State feed analyses as indications of suspect dairy rations.

Do not sample dairy products such as cheese or yogurt, unless there is reason to suspect they were made from milk containing violative levels of aflatoxin M_1.

g. When collecting a follow-up regulatory sample to a positive *surveillance* aflatoxin sample, record the sample number and ppb findings for the *surveillance* in the "Remarks" section of the new C/R.

2. **Patulin**

a. See Attachment A, Section 2. **Only** collect apple juice and/or concentrated apple juice. Since Patulin is not destroyed by heat processing, and can occur at high levels in apple juice, including pasteurized apple juice, if rotten, moldy or damaged apples are used to make the juice, both pasteurized and non-pasteurized single strength juice

and concentrated juices are to be collected.

If samples are collected in conjunction with the inspection of an apple juice processor, consider collecting samples if the processor does not cull apples to be used to produce juice (including stored apples) to remove rotten apples and visibly damaged apples i.e. bruising, breaks in the skin, holes, visible mold, hail damage, bird pecks. Consider collecting samples also if it is established that the processor uses drops i.e., apples that have fallen off the tree and are harvested from the ground, (also known as grounders or ground fruit) to produce juice.

b. Surveillance sample size is dependent on product form.

Frozen Concentrate:	Collect six subsamples with a minimum volume of 400 mL (approximately 12 fluid ounces) per subsample;
Single Strength (ready to drink):	Collect six subsamples with a minimum volume of 500 mL (approximately 16 fluid ounces) per subsample

If necessary, collect additional units to make up minimum volume requirements.

c. When collecting a regulatory sample as a follow-up to a positive surveillance patulin sample, record the sample number and ppb findings for the surveillance in the "Remarks" section of the new C/R.

d. Regulatory sample size is dependent on product form

Frozen Concentrate:	Collect six subsamples with a minimum volume of 400 mL (approximately 12 fluid ounces) per subsample;
Single Strength (ready to drink):	Collect six subsamples with a minimum volume of 500 mL (approximately 16 fluid ounces) per subsample

If necessary, collect additional units to make up minimum volume requirements.

If retail packaged samples are collected, then separate 702(b) samples must also be collected.

3. Surveillance Sampling for **Deoxynivalenol (DON)**

See Attachment A, Section 4 for products to be collected.

Surveillance samples will consist of four (4) 450 gram (approximately 1 pound) subsamples to be collected from a single lot of product.

4. Surveillance Sampling for **Fumonisin FB_1 and FB_2**

See Attachment A, Section 3 for products to be collected.

* For data base requirements, CFSAN needs approximately half of the samples to be degermed, i.e., low fat. Therefore, the investigator should try to collect half of samples of whole or partially degermed dry-milled corn products (fat content greater than or equal to 2.25%, dry weight basis) **AND** for the other half, degermed dry-milled corn products (fat content less than 2.25%, dry weight basis) of corn flour, corn meal, corn grits and corn flaking grits. Because of this requirement, these products should be collected at a mill if possible; however, major processors using these products, will have fat content as one of the specifications and if the investigator can determine the approximate fat content, samples can be collected from processors. State on the collection report, in the remarks section, that the product is either whole, partially degermed or degermed and state the approximate fat content obtained from either the miller or processor.

Collect ten 454-gram (one pound) subsamples per sample.

5. Surveillance Sampling for **Ochratoxin A**

See Attachment A, Section 5 for products to be collected. For cereals and soya based baby food products, collect 4 (four) 200 gram (Approximately 8 ounces) subsamples per sample, for all other products collect *4 (four)* 450 gram (approximately one pound) subsamples per sample.

C. Sample Handling: DO NOT pack samples (other than milk or fluid items) in plastic bags or other moisture-proof containers as this may cause sweating and result in an unstable sample. Refer to IOM, Chapter 4.

D. Sample Submission

Each FY, consult the ORA Field Work Plan, Part 1, Appendix 3 to determine where all mycotoxin samples should be sent. At the time this program is being written, the following analyzing laboratories are correct; however, if there is a conflict after the work plan is issued, the workplan is the lead document.

1. All regions should submit samples for *surveillance and regulatory* **aflatoxin analysis** to SRL at the following address:

U. S. Food and Drug Administration
Southeast Regional Laboratory (HFR-SE600)
60 Eighth Street, NE
Atlanta, GA 30309

2. All regions should submit samples for **fumonisin analysis** to the KAN-DO Lab at the following address:

U. S. Food and Drug Administration
Kansas City Laboratory (HFR-SW360)
11630 West 80th Street
Lenexa, KS 66214-3338

3. All regions should submit samples for **deoxynivalenol, ochratoxin and patulin analysis** as per the following table:

Region	Deoxynivalenol laboratory	ochratoxin laboratory	patulin laboratory
Northeast	NRL	NRL	NRL
Southeast	SRL	KAN	SRL
Central (BLT, CIN, NWJ, PHI)	NRL	NRL	NRL
Central (CHI, DET, MIN)	SRL	KAN	SRL
Southwest	KAN	KAN	KAN
Pacific	PRL-NW	PRL-NW	PRL-NW

PART IV - ANALYTICAL

A. Analyzing Laboratories:

Each FY, consult the current year ORA Field Work Plan, Part 1, Appendix 3 to determine where aflatoxin, fumonisin, deoxynivalenol, ochratoxin A and patulin surveillance samples will be analyzed. At the time this document is being written, the following information is current. If when this information differs from that published in the ORA Field Work Plan, the Field Work Plan is the lead document.

Laboratory	Analyzes for:
Kansas City District Lab (KAN)	Fumonisin, DON, Ochratoxin, Patulin
Northeast Regional Lab (NRL)	Aflatoxin, DON, Ochratoxin, Patulin
Pacific Regional Lab-NW (PRL-NW)	DON, Ochratoxin, Patulin
Southeast Regional Lab (SRL	Aflatoxin, DON, Ochratoxin, Patulin

B. Aflatoxin Analyses

SRL is responsible for all routine aflatoxin analysis. NRL is responsible for confirmation of aflatoxin identity by negative chemical ionization mass spectrometry, as required. SRL may, at its discretion, perform the confirmation analysis instead of submitting it to NRL.

SAFETY: Be aware of the potential hazards in the preparation of aflatoxin samples. See Section 49.2.01 (AOAC Method 977.16), 17th Edition of the Official Methods of Analysis of the AOAC.

1. Follow the procedures and methods in the 17th Edition of the Official Methods of Analysis of the AOAC:

   a. Chapter 49 - General precautions

   b. Section 49.2.01 (AOAC Method 977.16) - Sampling and preparation of sample and safety precautions.

   c. Sections 49.2.02 (AOAC Method 970.44) and 49.2.03 (AOAC Method 971.22) - Preparation of standards

   d. Section 49.2.04 (AOAC Method 975.35) - Identification of aflatoxins by TLC - Alternative Developing Solvents

   e. Section 49.2.08 (AOAC Method 968.22) - Method I (CB Method) for peanuts/peanut products (applicable to all nut products in this program)

   f. Section 49.2.09 (AOAC Method 970.45) - (BF Method) - Aflatoxins in peanuts and peanut butter

   g. Section 49.2.10 (AOAC Method 991.15) - Total aflatoxin levels in peanut butter

   h. Section 49.2.11 (AOAC Method 971.23) - Aflatoxins in cocoa

beans

i. Section 49.2.12 (AOAC Method 971.24) Aflatoxins in coconut, copra, and copra meal

j. Section 49.2.15 (AOAC Method 993.17) - Aflatoxins in corn and peanuts

k. Section 49.2.17 (AOAC Method 990.33) - Corn and peanut butter

l. Section 49.2.18 (AOAC Method 991.31) - Corn, raw peanuts and peanut butter.

m. Section 49.2.19 (AOAC Method 980.20) - Aflatoxins in cottonseed products

n. Section 49.2.19A (AOAC Method 994.08) - Corn, almonds, Brazil nuts, peanuts, and pistachio nuts.

o. Section 49.2.26 (AOAC Method 975.37) - Identification of the aflatoxin B1 by derivative formation on TLC plate.

p. Section 49.2.27 (AOAC Method 985.17) - Identification of aflatoxin B_1, confirmation method

q. *Section 49.2.29 (AOAC Method 999.07) - Aflatoxins in peanut butter, pistachio paste, fig paste, and paprika - immunoaffinity column/LC with post column derivatization. *

r. Section 49.3.01 (AOAC Method 974.17) - Aflatoxins M_1 in dairy products

s. Section 49.3.02 (AOAC Method 980.21) - Aflatoxin M_1 in milk and cheese; confirmation of identity by derivative formation on TLC plate

*AOAC methods that are not in the 17th edition of Edition of the Official Methods of Analysis of the AOAC:

t. AOAC Method 2000.16 Aflatoxin in baby food, immunoaffinity column cleanup and LC (J.AOAC Int. 2001 in press)

u. AOAC 2000.18 Aflatoxin M_1 in liquid milk, immunoaffinity column/LC (Dragacci, S. Grosso, F., and Gilbert, J., "Immunoaffinity column cleanup with liquid chromatography for determination of aflatoxin M_1 in liquid milk: collaborative study", J. AOAC Int. 84: 437-443, 2001) *

The chart below may be used to facilitate calculations for nut samples.

Nuts	Meat, % by Wt.
Almonds	40
Peanuts	70
Pecans	50
Pistachios	50
Macadamia	29
Pumpkin seeds	74
Walnuts	50

Do not conduct a check analysis on *surveillance* samples.

If any *surveillance* sample is positive (any level), use lab class "2" and immediately arrange for collection of a regulatory sample which is representative of the lot. *Use class "1" for a surveillance sample only if * no aflatoxin is detected.

2 Confirmation .

Refer to Compliance Policy Guides for required confirmation of identity procedures for regulatory samples. Mass spectral confirmation of aflatoxin identity is not required of domestic peanuts/peanut products, corn/corn meal, pistachio nuts, pumpkin seeds, cottonseed/meal, coconut meal, or copra. When aflatoxin levels exceed the guideline in any other product:

- wrap the vial containing the aflatoxin B1 in foil to protect the contents against light and moisture;
- *after notifying the confirmation lab of its transmittal, send the vial by a one-day delivery service either to NRL (HFR-NE580; 158-15 Liberty Avenue, Jamaica, NY 11433; (718) 622-5439).
- Note: At the option of the lab doing the initial analysis, that lab may also do the confirmation analysis instead of sending it to NRL. *

Confirmation of identity will be by negative ion chemical ionization mass spectrometry as per B.1.s.above. In Part VI, references #3 and #4 pertain to this method.

3. Reporting

The analyzing district will report analytical results into FACTS using the LMS codes in the "LMS Code Manual". It is particularly important that all analysis be entered using the flag code "MYC" and that the correct Mycotoxin Code **for the mycotoxins analyzed for** be used.

For follow up regulatory samples, report the sample number and ppb findings for the *surveillance* samples in the narrative field of the FDA 2196(b).

C. Patulin Analyses

1. General: Samples of frozen concentrate should be diluted either as per recommendation for dilution or to a Brix value of 11.5 (single strength) before analysis (Federal Register 56 No. 127, pp30452-30466, 1991).

2. Methods:

Follow the procedures and methods in the 17th Edition of the Official Methods of Analysis of the AOAC:

Section 49.7.02 AOAC Method 995.10 - Patulin in apple juice, liquid chromatographic method, AOAC-IUPAC-IFJU Method. AOAC International adopted this method in 1995. The method is published in JAOAC 79(2): 451-455, 1996.

AOAC method that is not in the 17th edition of Edition of the Official Methods of Analysis of the AOAC:

AOAC method 2000.02 Patulin in clear and cloudy apple juices and apple puree (McDonald, S., Long, M., and Gilbert, J., "Liquid chromatographic method for determination of patulin in clear and cloudy apple juices and apple puree: collaborative study", J.AOAC Int. 83:1387-1394)

3. Confirmation of Identity of Patulin:

For regulatory samples, confirmation is to be done by mass spec. Two acceptable procedures are:

Rupp, H.S., Turnipseed, S.B. "Confirmation of patulin and 5-hydroxymethylfurfural in apple juice by gas chromatography/mass spectrometry", J. AOAC Int. 83: 612-626, 2000)

Roach, J.A.G., White, K.D., Trucksess, M.W., and Thomas, F.S., "Capillary gas chromatography/mass spectrometry with chemical ionization and negative ion detection for confirmation of identity of patulin in apple juice", J. AOAC Int. 104-112, 2000)

4. Reporting *

The analyzing district will report analytical results into FACTS using LMS codes from the "LMS Code Manual". It is particularly important that all analysis be entered using the flag code "MYC" and that the correct Mycotoxin Code **for the mycotoxins analyzed for** be used.

D. Deoxynivalenol Analyses (DON)

The method of analysis is published in J AOAC Intl. 79(4):883-887, 1996. This is the same method used in previous deoxynivalenol field assignments.

The analyzing district will report analytical results into FACTS using LMS codes from the "LMS Code Manual". It is particularly important that all analysis be entered using the flag code "MYC" and that the

correct Mycotoxin Code **for the mycotoxins analyzed for** be entered into field position 25 as per Appendix I.
If DON is not found, the sample should be assigned a Lab Class "1". If it is found at any level, it should be assigned a Lab Class "2".

*Notify the Domestic Mycotoxin Monitor, Robyn Jones by e-mail when the Deoxynivalenol guidance level of 1 ppm is exceeded. *

E. Fumonisin Analyses

*Follow the procedures and methods in the 17th Edition of the Official Methods of Analysis of the AOAC:

Section 49.5.01 * AOAC Method 995.15 - Fumonisins FB_1, FB_2, and FB_3 in corn, liquid chromatographic method, AOAC-IUPAC method. This method was adopted by AOAC International in 1995, and is published in the J. AOAC Intl. 79(3): 688-696, 1996. The method was developed specifically for corn, therefore for products other than corn; it is recommended that recovery studies be done on such products before the final analyses.

*AOAC method that is not in the 17th edition of Edition of the Official Methods of Analysis of the AOAC:

AOAC Method 2001.04 Determination of fumonisin FB_1 and FB_2 in flour and corn flakes by LC with immunoaffinity column cleanup(J. AOAC Int., in press 2001)*

The analyzing district will report analytical results into FACTS using LMS codes from the "LMS Code Manual". It is particularly important that all analysis be entered using the flag code "MYC" and that the correct Mycotoxin Code **for the mycotoxins analyzed for** be used.

If Fumonisin is not found, the sample should be assigned a Lab Class "1". If it is found at any level, it should be assigned a Lab Class "2".

*Notify the Domestic Mycotoxin Monitor, Robyn Jones by e-mail when the guidance levels (listed in Part I.B.4) for total fumonisins (FB_1 + FB_2 + FB_3) are exceeded. *

F. Ochratoxin A Analyses

The method of analysis is the AOAC Method 991.44, Ochratoxin A in Corn and Barley. The method was published in JAOAC 79:1102 1996. This method was modified and a copy of the modified method was supplied to KAN, and SEA.

* (AOAC method not in the 17th edition of Edition of the Official Methods of Analysis of the AOAC) AOAC method 2000.03 Ochratoxin A in barley, immunoaffinity column/LC (Entwisle, A.C., Williams, A.C., Mann, P.J., and Slack, P.T., "Liquid chromatographic method with immunoaffinity column cleanup for determination of ochratoxin A in barley: collaborative study", J. AOAC Int. 83:1376-1383, 2000) *

The analyzing district will report analytical results into FACTS using LMS codes from the "LMS Code Manual". It is particularly important that all analysis be entered using the flag code "MYC" and that the correct Mycotoxin Code **for the mycotoxins analyzed for** be used.

If ochratoxin is not found, the sample should be assigned a Lab Class "1". If it is found at any level, it should be assigned a Lab Class "2".

*Notify the Domestic Mycotoxin Monitor, Robyn Jones by e-mail when an Ochratoxin A level greater than **4 ppb** is found in any commodity.. A maximum level of 5 ppb has been proposed as an international standard by CODEX for ochratoxin A in wheat, barley, rye, and their derived products; therefore data is needed from this Compliance Program to support the U.S. position on this issue. *

PART V - REGULATORY/ADMINISTRATIVE STRATEGY

A. Aflatoxin

The following sections of the Compliance Policy Guides are applicable when recommending legal actions against products collected under this program:

Section 527.400 **Whole Milk, Low Fat Milk, Skim Milk - Aflatoxin M_1** (CPG 7106.10)
Section 570.200 **Brazil Nuts - Adulteration with Aflatoxin** (CPG 7112.07)
Section 570.375 **Aflatoxin in Peanuts and Peanut Products** (CPG 7112.02)
Section 570.500 **Pistachio Nuts - Aflatoxin Adulteration** (CPG 7112.08)
Section 555.400 **Foods, Adulteration with Aflatoxin** (CPG 7120.26)

The following MOUs with USDA are in effect:

Peanuts and Peanut Products:	225-96-2001
Brazil Nuts:	225-96-2002
Pistachio Nuts:	225-96-2003

Complete copies of the MOUs can be obtained by contacting the Division of Compliance Policy, Office of Enforcement, HFC-230, at (301) 827-0420.

Mass spectral confirmation of aflatoxin identity is required for all domestic foods except peanuts/peanut products, corn/corn meal, pistachio nuts, pumpkin seeds, cottonseed/meal, coconut meal, and copra. Aflatoxin identity of all samples should be confirmed using AOAC method 975.37

The Home District must report analytical results on regulatory samples that are "out of compliance" (i.e., judged to be of regulatory significance) to the responsible firm and to cooperating State officials within their Districts.

When milk samples contain more than 0.5 ppb of aflatoxin M_1, dairy feed should be sampled under the appropriate Center for Veterinary Medicine (CVM) programs to determine the source of the contamination. Initiate appropriate legal action against dairy products under this program, and against feed under CVM's instructions.

FDA has agreed that aflatoxin contaminated peanuts may be processed into oil since normal refining effectively removes aflatoxins present in the crude oil. The residual meal cannot be used for domestic food or feed unless shown by analysis to be at a level for aflatoxins that is not of regulatory significance.

Immediately notify the Office of International Affairs, HFG-1, at (301) 827-4480, when informed of export lots of corn identified by USDA as appearing to be actionable, so that appropriate follow-up can be initiated.

Districts with recurrent aflatoxin problems within their boundaries should consider conducting aflatoxin control workshops. Assistance (workshop materials, speakers on selected topics, etc.) in developing such programs is available through Industry Activities Staff, HFS-565 (202) 205-5251.

B. Patulin

*The following Compliance Policy Guide is applicable when recommending legal actions against products collected under this program:

Section 510.150: Apple Juice, Apple Juice Concentrates, and Apple Juice Products - Adulteration with Patulin

Mass spectral confirmation of patulin identity is required for all domestic and import samples.

C. **Fumonisin, Deoxynivalenol, Ochratoxin A**

CFSAN will use the data on the incidence and level of these mycotoxins in various commodities in conjunction with toxicological data:

- to access the need for guidance and/or enforcement levels;
- to conduct risk assessment;
- to address international concerns;
- to initiate discussions with industry;
- and to conduct any follow up action necessary for Public Health Protection. *

附件六

真菌之毒性檢驗

I Brine shrimp 之培養

中央有橫隔之培養皿（橫隔有小孔隙可供 shrimp 游過，圖一）

↓培養皿中加入 0.85% 之鹽水

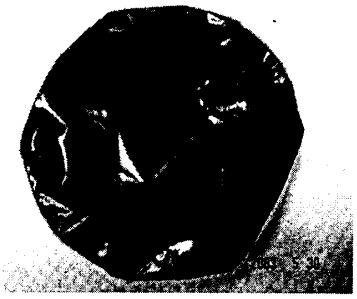
↓於培養皿一邊加入 shrimp egg

↓用已挖洞之黑色紙將培養皿蓋住，洞孔位於未加入 shrimp egg 之一邊（圖二）

↓孵化之 shrimp 會向光而游向培養皿之另一端

取成熟之 shrimp 供檢驗

圖一：培養皿中間之橫隔有孔洞，可供成熟之 brine shrimp 游過。



圖二：已挖洞之黑色紙將培養皿蓋住，洞孔位於未加入 shrimp egg 之一邊，孵化成熟之 brine shrimp 有向光性，故會游向有光的另一端。

II 真菌培養及毒素萃取

將真菌於三角錐瓶中培養（培養基為 G25N； 25% 甘油+75% 含氮有機物）

↓將長徽之培養基於三角錐瓶切碎

↓以甲醇溶液中浸泡一夜

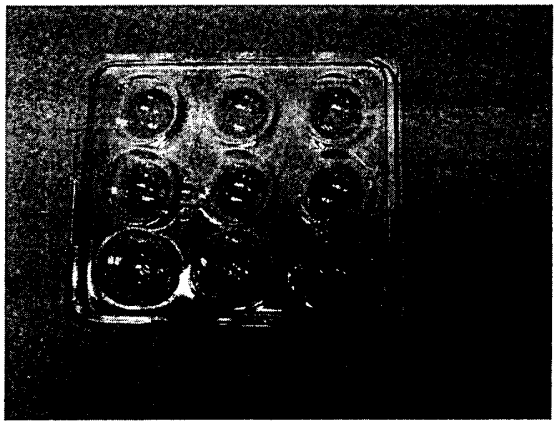
↓過濾

↓加不同量之濾液加於多槽之培養皿上(圖三)

↓讓甲醇揮發乾

↓加入 brine shrimp 測試

如 brine shrimp 死亡，則表真菌可能會產生毒素



圖三：多槽之培養皿。

附件七

黃麴毒素（Aflatoxins）之檢驗

I. Pre-column-TFA（三氟醋酸；以下簡稱 TFA）

1. TFA 衍生液之製備：

黃麴毒素標準品溶於 0.5 mL 之 Acetonitrile

↓氮氣吹乾

↓分別加入TFA 0.1 mL進行衍生化

↓混勻後置於室溫下15分鐘

↓加入丙酮與水(9:1)混合液0.9 mL

黃麴毒素標準品TFA衍生液

2. 儀器設備：

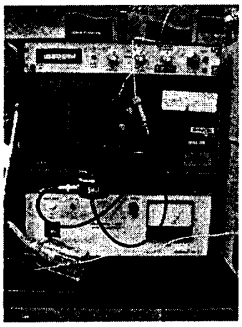
層析管柱：C-18, 3um , 150x4.6 mm ID , YMC-AQ-302-3

Pump：Waters 600

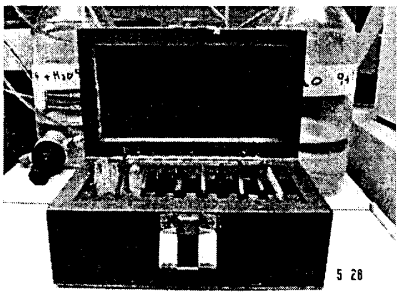
Autosampler：Shimadzu Sil 10A

Spectrofluorescence detector： Mcpherson Instrument FL-750 BX

註：Mcpherson Instrument 之 spectrofluorescence detector（圖四）利用有色玻璃片（Cut off filter；圖五）將光隔離，例如本實驗就利用玻璃片將波長低於 418 nm 之發射光隔離。據 FDA 人員表示，此螢光偵測器靈敏度高，故選用此機種。



圖四：Spectrofluorescence detector



圖五：各式之 Cut off filter

3. 層析條件：

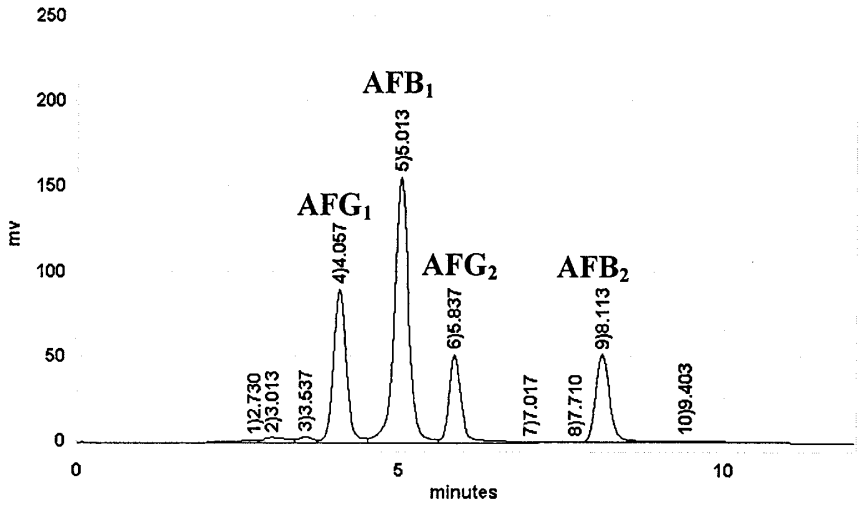
注射量：10 μL

流速：1 mL/min

移動相：50 %甲醇水溶液

螢光偵測器：激發光 335 nm，發射光 ≥418 nm

4. 層析結果：



II. 使用 PHRED（Photochemical Reactor For Enhanced Detection）

1. 標準品配置：

黃麴毒素標準品溶於 40% acetonitrile(先將黃麴毒素標準品溶於少量 acetonitrile 後再加水，因黃麴毒素對水的溶解度不佳)。

2. 儀器設備：

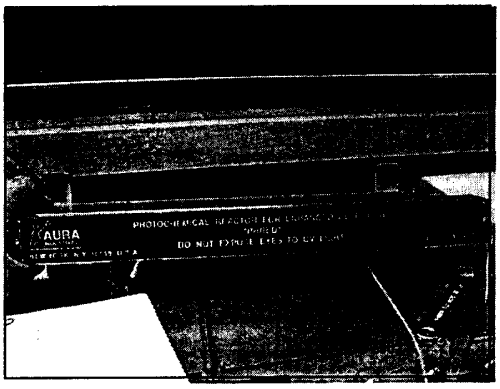
層析管柱：C-18, 3um , 150*4.6 mm ID , YMC-AQ-302-3

Pump：Waters 600

Autosampler：Shimadzu Sil 10A

Spectrofluorescence detector： Mcpherson Instrument FL-750 BX

Photochemical Reactor For Enhanced Detection（圖六）



圖六：Photochemical Reactor For Enhanced Detection

3. 層析條件：

注射量：10 μL

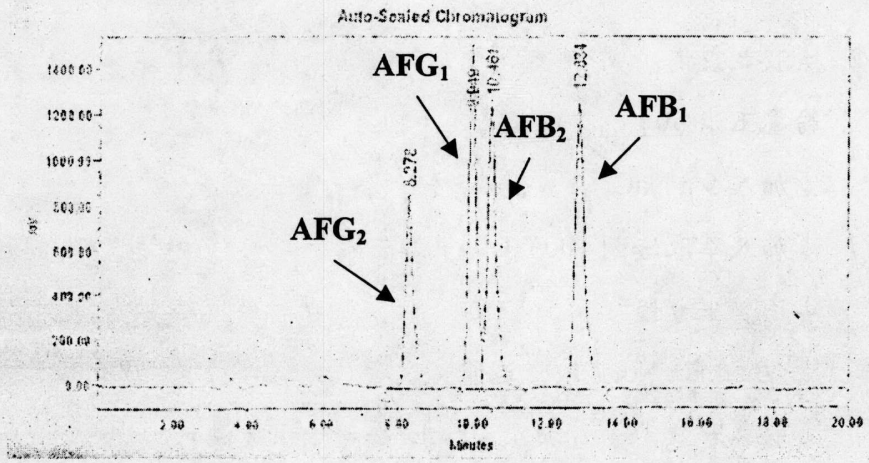
流速：1 mL/min

移動相：乙腈＋水＋甲醇＋醋酸=125：300：62：2.5 (v/v/v)

螢光偵測器：激發光＝333 nm，發射光 ≥ 418 nm

4. 層析結果：

Sample Name	AFLATOXIN 5ng/ml	Sample Type	Unknown
Vial	1	Date Acquired	5/27/03 2:03:44 PM
Injection	1	Acq Method Set	MIKE
Injection Volume	50.00 ul	Processing Method	mike
Channel	SATIN	Date Processed	5/27/03 2:23:57 PM
Run Time	20.0 Minutes		



附件八

伏馬毒素（Fumonisins）之檢驗

1. 標準品配置：

將 FB_1、FB_2 及、FB_3 之標準品以乙腈–去離子水溶液 (1：1，v/v) 稀釋

2. 檢液之製備

粉墨玉米 50 g

↓ 加入 5 g NaCl

↓ 加入萃取溶劑 100 mL（甲醇：水＝4：1 (v/v) 體積比例混合）

↓ 均質 1 分鐘

↓ Whatman No.4 濾紙過濾

↓ 取萃取液 10 mL 加入 40 mL 去離子水後混勻

↓ 混合液通過玻璃纖維濾

↓ 量取 2 mL

↓ 以 1 滴/秒之流速通過 FumoniTest™（Vicam）免疫親和性管柱

↓ 去離子水 5 mL 清洗兩次

↓ 以 1.5 mL 甲醇 流出 (1~2 滴/秒)

↓ 氮氣吹乾（因為甲醇會使 peak 變寬）

↓ 加入 200 μL 之乙腈：水＝4：1 (v/v)

檢液

3. 衍生化

a. 衍生化試劑：

鄰-苯二甲醛試劑 (以下簡稱 OPA 試劑；取鄰-苯二甲醛 40 mg 溶於甲醇 1 mL，續以 0.1 M 四硼酸二鈉溶液 5 mL 稀釋，再加入乙硫醇 50 μL 混勻，即為 OPA 試劑，需於一週內使用)

b. 利用 Shimadzu Sil 10A autosampler 之自動混合功能將檢液（50 μL）及衍生化試劑（200 μL）混合並注入（10 μL），混合至注入的時間約 2 分鐘。

4. 儀器設備：

層析管柱：C-18, 3um , 150x4.6 mm ID , YMC-AQ-302-3

Pump：Waters 600

Autosampler：Shimadzu Sil 10A

Spectrofluorescence detector： Mcpherson Instrument FL-750 BX

5. 層析條件：

注射量：10 μL

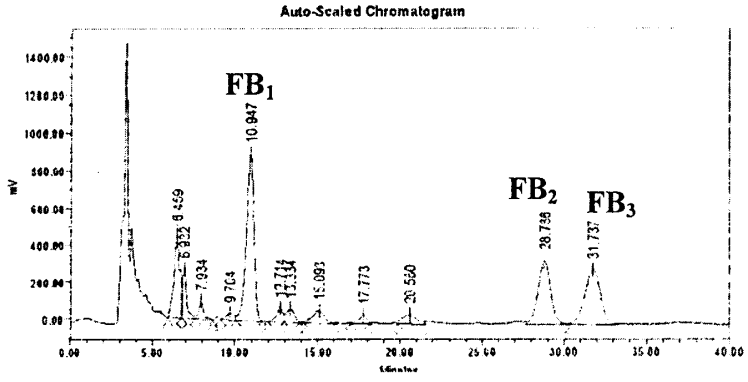
流速：1 mL/min

移動相：乙腈＋水＋醋酸＝500：500：10 (v/v/v)

螢光偵測器：激發光＝333 nm，發射光 ≥ 418 nm

6. 層析結果：

SampleName	FB Stds	Sample Type	Unknown
Vial	1	Date Acquired	5/29/03 10:34:12 AM
Injection	1	Acq Method Set	MIKE
Injection Volume	50.00 ul	Processing Method	mike
Channel	SATIN	Date Processed	5/29/03 11:14:25 AM
Run Time	40.0 Minutes		



附件九

黃麴毒素（Aflatoxin）及
赭麴毒素（Ochratoxin A）之檢驗

1. 赭麴毒素標準品配置：

   赭麴毒素為酸性，先用乙腈–醋酸溶液（99:1）溶解，再加 40% 乙腈水溶液稀釋。

2. 儀器設備

   層析管柱：C-18, 3um , 150*4.6 mm ID , YMC-AQ-302-3

   Pump：Waters 600

   Autosampler：Shimadzu Sil 10A

   spectrofluorescence detector： Mcpherson Instrument FL-750 BX

3. 層析條件：

   注射量：10 μL

   流速：1 mL/min

   移動相 A：乙腈＋甲醇＋水＝250：125：600 (v/v/v)

   移動相 B：乙腈＋水＋醋酸＝600：400：5 (v/v/v)

   螢光偵測器（黃麴毒素）：激發光 365 nm，發射光 ≥418 nm

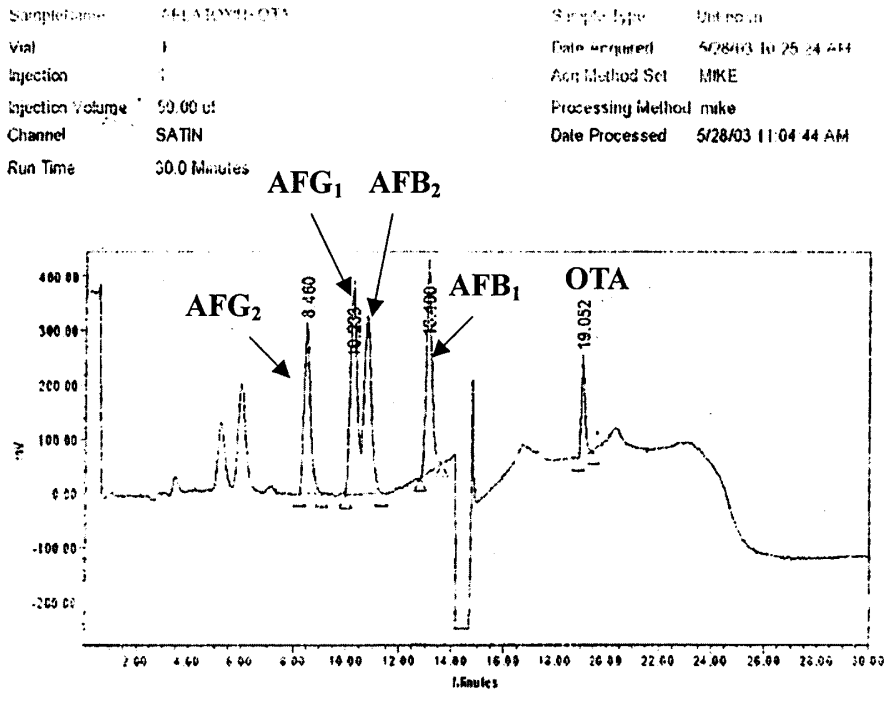
   螢光偵測器（赭麴毒素）：激發光 333 nm，發射光 ≥418 nm

4. Gradient Program

Time	Flow	% A	% B	螢光偵測器
Initial	1.00	100	0	365 nm
5.00	1.00	100	0	365 nm
10.00	1.00	0	100	333 nm
18.00	1.00	100	0	333 nm

註：每次完成須以移動相 A 平衡 10 min 再進行下次層析。

5. 層析結果：



附件十

棒麴毒素（Patulin）之檢驗

1. 原理

   a. 棒麴毒素以 ethyl acetate 萃取後利用 sodium carbonate solution 純化。

   b. 萃取液利用 anhydrous sodium sulfat 將 ethyl acetate 吹乾。

   c. 棒麴毒素 reversed-phase LC with UV detection 分析。

2. 標準品配置：

   棒麴毒素標準品以乙醇稀釋。

3. 檢液之製備

   Glass tube A (20 x 150 mm, glass with cap)

   ↓ 5 mL juice + 10 mL ethyl acetate

   ↓ shake 1 min

   ↓ let layers separate

   ↓ transfer upper layer to another tube B

   ↓ add 10 mL ethyl acetate to tube A

   ↓ shake 1 min

   ↓ let layers separate

   ↓ transfer upper layer to tube B

   ↓ add 2 mL Na2CO3 solution to tube B

   ↓ shake, let layers separate

   ↓ transfer upper layer to tube C

   ↓ add 5 mL ethyl acetate to tube B

   ↓ shake, let layers separate.

↓ transfer upper layer to tube C

↓ add 1 g anhydrous Na_2SO_4 to tube C

↓ shake, let solids settle

↓ decant solution into tube D (or flask)

↓ place tube D (or flask) in water bath at 40℃ under N2

↓ evaporate to 1-2 mL

↓ quantitatively transfer extract to a 8 mL vial

↓ rinsing tube 3 x 1 mL ethyl acetate

↓ evaporate to dryness

↓ dissolve residue in 0.5 mL pH 4 acetic acid-water

檢液（store test solution in freezer if not analysis immediatly）

4. 儀器設備：

層析管柱：3.9 x 150 mm， 5 um，10% carbon load

層析儀：SpectraSystem P4000

5. 層析條件：

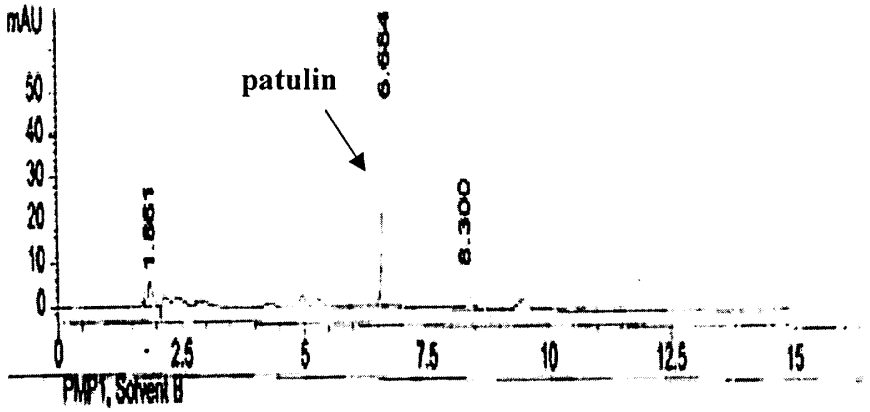
注射量：10 μL

流速：1 mL/min

移動相：0.8 mL tetrahydrofuran + 100 mL water

UV 偵測器：波長 276 nm

6. 層析結果：


mAU
50
40
30
20
10
0
patulin
6.684
1.861
6.300
0
2.5
5
7.5
10
12.5
15
PMPT, Solvent B

附件十一

DON 之檢驗

1. 標準品配置：

DON 之標準品以甲醇稀釋（DON 可穩定保存於甲醇及乙腈溶液）

2. 檢液之製備

25g 玉米

↓加 50 mL 之萃取液(乙腈：水=9：1)

↓攪拌混勻 3 min

↓Whatman No.4 濾紙過濾

↓量 6 mL 濾液 to 15mL polypylene centrifuge tube（圖七）

↓將 clean up column (Mycosep 225 column)置於 polypylene centrifuge tube 之另一端（圖八）

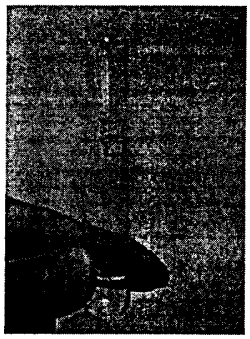
↓緩慢的將 clean up column (rubber flange end)推 polypylene centrifuge tube（圖九）

↓取通過 clean up column 之濾液 2 mL

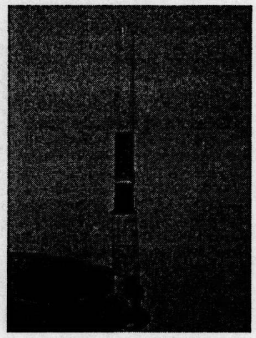
↓氮氣吹乾

↓溶於 50% 甲醇

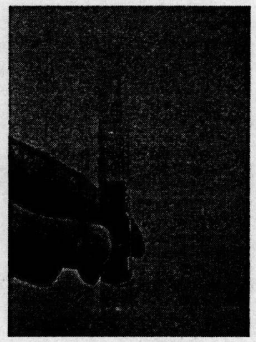
檢液



圖七：濾液加入 polypylene centrifuge tube



圖八、clean up column 置於 polypylene centrifuge tube 之另一端



圖九、緩慢的將 clean up column 推 polypylene centrifuge tube，過濾後之濾液位於上層。

3. 儀器設備：

層析管柱：Becman ODS, 5 um , 250*4.6 mm ID

層析儀：SpectraSystem P4000

4. 層析條件：

注射量：10 μL

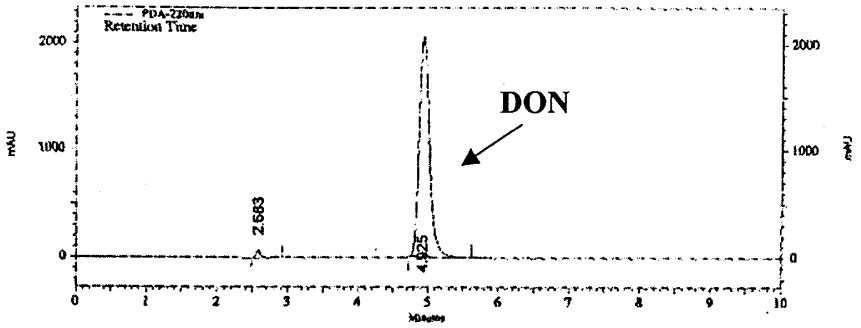
流速：1 mL/min

移動相：甲醇–水溶液（依層析圖的雜訊多寡而定，甲醇比例越高，DON peak 出現的越早，但分離效果越差）

UV 偵測器：波長 254 nm

5. 層析結果：

Analyst: System
Sample ID: DON-300 **Vial: A04** **Injection Volume: 10**



附件十二

Elisa test for T-2 Toxin

1. Elisa test kit（Veratox）

Includes antibody well stripes, red mixing well strips, blue-labeled bottle, red-labeled bottle, green-labeled bottle, calibration standards （0ppb, 25ppb, 50ppb, 100ppb and 250ppb）

2. Sample preparation

mix sample till it pass 20 mesh sieve

↓ 5 g sample + 25 mL 50% methanol

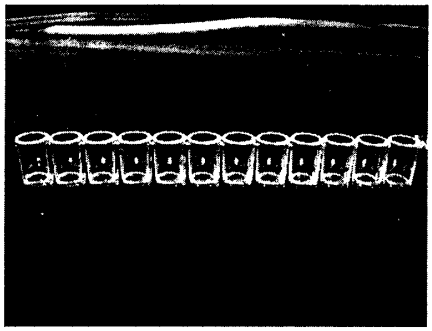
↓ shake vigorously for 3 min

↓ filter at least 5 mL through a whatman#1 filter

collect the filtrate as sample

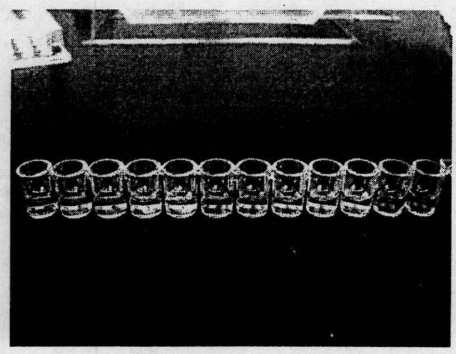
3. Quantitation test

a. Add 100 μL of conjugate from the blue-labeled bottle in each red-marked mixing well.



b. Transfer 100 μL of calibration standards （0ppb, 25ppb, 50ppb, 100ppb and 250ppb） and samples to the red-marked mixing wells.

c. Use the 12-channel pipette, mix the wells by pipetting it up and down 3 times.

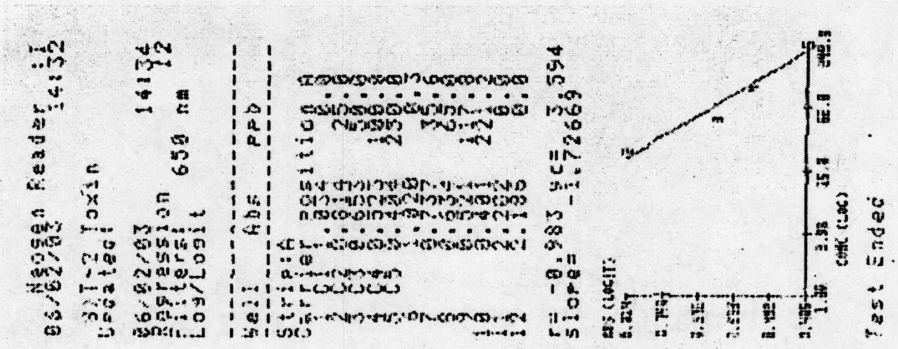


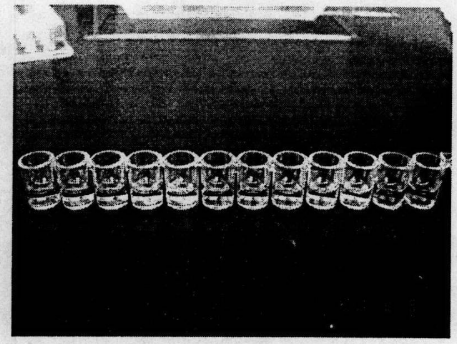
n. Pipette the red stop solution from the red-labeled reagent boat.

o. Add 100 μL red-labeled reagent to each well and mix by sliding back and forth on a flat surface.

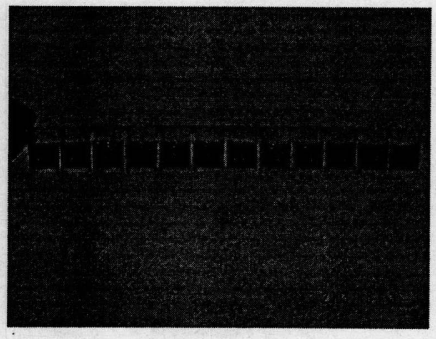
p. Wipe the bottom of microwells with dry cloth or towel and read a microwell reader using 650nm（result should be read within 20 min after adding red reagent）.

4. Results





14. Pipette the red stop solution from the red-labeled reagent boat.
15. Add 100 μL red-labeled reagent to each well and mix by sliding back and forth on a flat surface.



16. Wipe the bottom of microwells with dry cloth or towel and read a microwell reader using 650nm(result should be read within 20 min after adding red reagent) .

4. Results