出國報告（出國類別：國際會議）

參加由 SELAMAT 舉辦之「真菌毒素分析與風險評估研習會」

服務機關：行政院衛生署藥物食品檢驗局
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派赴國家：中國
出國日期：95 年 12 月 10 日至 14 日
報告日期：96 年 1 月 30 日

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壹、摘　要

SELAMAT於95年12月11至13日於中國北京舉辦「真菌毒素分析及風險評估研習會」，此次會議由中國北京「中國農業科學院植物保護研究所」負責辦理，派員參加之國家有日本、泰國、台灣、馬來西亞、印尼、菲律賓、荷蘭、南韓、英國、土耳其、新加坡及主辦國中國等十二國。

赴中期間學習真菌毒素基本特性、各國之限量標準、分析方法之品質保證、樣品前置備處理(淨化步驟)、風險評估、常用之分析儀器設備介紹及檢驗技術未來之導向等。目前用來作為真菌毒素快速篩選的方法有固相淨化管柱(Solid Phase Clean Up Columns)、免疫親和管柱(Immunoaffinity Columns)、酵素連結免疫吸附分析法(ELISA)、Membrane Cards及試紙條檢測(Lateral Flow Devices)。在此次研習會中，學習到利用R-Biopharm真菌毒素快速檢測套組之實驗方法，與分別利用免疫親和管柱及固相淨化管柱進行樣品前處理之步驟，再利用高效液相層析質譜儀(LC/MS)、氣相層析質譜儀(GC/MS)及高效液相層析儀(HPLC)三種不同儀器分別進行檢測之標準操作流程。

職此次奉派參加此研討會，得以瞭解國際對於真菌毒素之檢驗方法，與我國正進行者相比較，我國真菌毒素檢驗方法均與國際相當，所得之資料也將有利於與國際接軌。亦與各國專家學者建立順暢的資訊互惠交流管道，例如英國中央科學實驗室(Central Science Laboratory)的 John Gilbert，荷蘭食品安全研究所(RIKILT-Institute of Food Safety)的 Wim Traag、Hans Marvin 及 Piet Stouten、韓國之食品工業發展研究所(Korea Food Research Institute, KFRI)的 Minseon Koo、新加坡農獸局(Agri-Food and Veterinary Authority, AVA)的李秀連小姐、日本食品安全總合研究所的久城真代小姐等，作為本局掌握國際資訊的橋樑，對於本局日後取得各國對於真菌毒素領域的資訊上有莫大的實質助益。

貳、過　程

為瞭解各國在真菌毒素檢驗技術的發展趨勢，職於 95 年 12 月 10 日至 14 日赴中國北京「中國農業科學院植物保護研究所」(Chinese Academy of Agricultural Sciences (CAAS) Institute of Plant Protection)研習真菌毒素之檢驗技術，並與來自各國之專家學者相互交流檢驗技術。

每日行程如下：

第一天(12 月 10 日　星期日)

早上 09:25 自桃園機場搭乘國泰航空 CX 403 班機出發至香港機場，再搭乘中國國際航空 CA 102 班機前往中國北京，到北京首都機場時間為下午 4 點半。當晚住宿北京友誼賓館(Beijing Friendship Hotel)。

第二天(12 月 11 日　星期一)

地點：北京友誼賓館會議廳(為對外開放之研討會，約 60、70 人參加)

一、 宣布會議正式開幕並致歡迎詞(Official Opening of SELAMAT Workshop)：主持人為中國農業科學院植物保護研究所的彭于發先生。

二、 介紹 SELAMAT (Introduction to SELAMAT Project)：由 SELAMAT 之執行長 Dr. Hans Marvin (RIKILT)介紹 SELAMAT 之組織及其任務。

「SELAMAT」在馬來語的意思為「安全」，全名為「Safety enhancement of Edible products, Legislation, Analysis and Management with ASEM countries by mutual Training and research」，目前已參與此組織的國家有中國、葡萄牙、印度尼西亞、新加坡、日本、泰國、韓國、荷蘭、馬來西亞、英國、越南，我國也在其中。SELAMAT 主要為透過相互的培訓和研究，與歐洲國家一起提昇可實用產品之安全、立法、分析與管理，其目標為建設一個歐洲國家與歐亞會議國家在食品安全議題上的國際合作網絡。

三、 鐮刀菌毒素風險評估研究發展(Recent developments in the risk assessment of Fusarium toxins)：主講人為英國中央科學實驗室(Central Science Laboratory)的 John Gilbert。

主要介紹 Deoxynivalenol (DON)、Zearalenone (ZON)、T-2 毒素及 Fumonisins B_1、B_2、B_3 之毒性效應、風險評估，以及介紹歐洲食品安全管理局(EFSA)如何評估動物飼料中之 Fumonisins 毒素。JECFA 所建立真菌毒素暫定每日最高容許攝入量(Provisional Maximum Tolerable Daily Intake, PMTDI)，針對 4-Deoxynivalenol 之 PMTDI 為 1 μg/kg bw、T-2 及 HT-2 毒素為 0.06 μg/kg bw (指 T-2 及 HT-2 單獨或合計的量)、而 Fumonisins 為 2 μg/kg bw (指 B_1、B_2 及 B_3 單獨或合計的量)。Fusarium 毒素會對抑制人體的免疫系統、刺激免疫功能(像是過敏症)、免疫毒性(Trichothecenes 會抑制蛋白質合成)。

四、 關於食品與飼料中鐮刀菌毒素的法規和指引(Regulations and guidelines for Fusarium toxins in food and feed)：主講人為荷蘭公共衛生與環境國家研究院(RIVM)的 Hans van Egmond。

主要介紹影響制定真菌毒素法規之因素、有關fusarium毒素之法規、世界各國對於DON、ZON、T-2毒素及Fumonisins之限量標準，以及歐洲的發展。影響制定真菌毒素法規之因素包含了毒理學資料的獲得、調查分析資料的獲得、採樣與分析方法的獲得、針對某些特定食物之飲食習慣、與其他國家的貿易接觸以及充分的食物供給等因素。針對真菌毒素之風險評估，可做為參考的有JECFA出版的「Safety evaluation of certain mycotoxins in food」及EFSA出版的「Evaluation of certain mycotoxins in food」。在世界各國真菌毒素法規部分，則有FAO在2004年所發行的「2003年全世界食品和飼料真菌毒素法規」，共有英文、中文、法文及西班牙文四個版本可供參考。

五、 鐮刀菌毒素分析概況(Fusarium Toxin Analysis：an Overview)：主講人為英國中央科學實驗室(Central Science Laboratory)的 John Gilbert。

介紹 Fusarium 毒素之分析方法，方法的選擇、毒素的結構式、萃取及淨化方法的選擇及儀器設備。

六、 食品與飼料中鐮刀菌毒素發生情況(Occurrence of Fusarium Toxins in Food and Feed)：主講人為荷蘭公共衛生與環境國家研究院(RIVM)的 Hans van Egmond。

鐮刀菌毒素普遍存在多種食物和飼料中，且這些毒素通常在加工過程中相當穩定。其中最常被發現的毒素為 DON，主要被汙染食物為小麥。

七、 鐮刀菌毒素篩選檢測方法(Screening Methods for Fusarium Toxins) ：主講人為 R-Biopharm 公司的 Patrick J. L. Taylor。

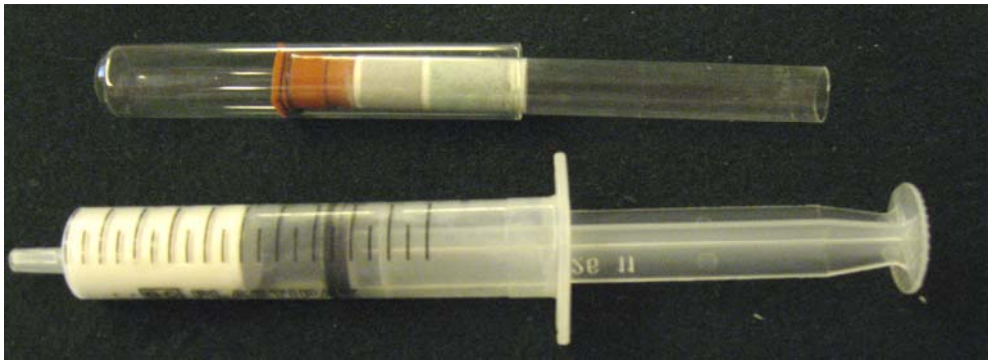
用來作為快速篩選的方法有固相淨化管柱(Solid Phase Clean Up Columns)、免疫親和管柱(Immunoaffinity Columns)、酵素連結免疫吸附分析法、Membrane Cards、試紙條檢測(Lateral Flow Devices)，R-Biopharm公司目前有針對以下真菌毒素的檢測方法：Aflatoxin B_1、B_2、G_1、G_2、M_1、M_2；Ochratoxin A、B；Citrinin；Trichothecenes；Zearalenone；Fumonisin B_1、B_2、B_3；DON；T-2及HT-2毒素。使用RIDASCREEN® FAST Mycotoxin Tests，實驗時間只需15分鐘，非常快速，若使用RIDA®QUICK試紙條檢測實驗，加入樣品後，只需等5分鐘即可知道結果，相當快速、準確，而且不需特別的儀器或設備，適用於工廠或戶外。

R-Biopharm公司產品之總表如下表：

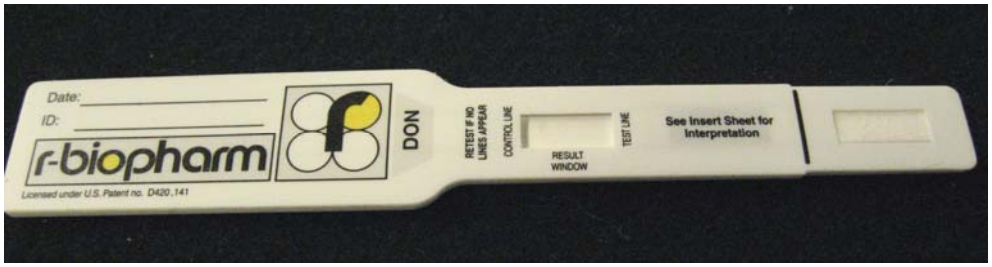
SCREENING TEST FORMATS			
RIDA®	RIDASCREEN®		
QUICK Tests	Mycotoxin Tests	FAST Mycotoxin Tests	
Aflatoxin	Aflatoxin Total	Aflatoxin M_1	Aflatoxin
DON	Aflatoxin B_1 30/15	DON	Aflatoxin SC
Fumonisin	Aflatoxin M_1 30/15	Fumonisin	Citrinin
		Ochratoxin A	
		T-2 Toxin	
		Zearalenone	
15 - 20 min	up to 2.5 hrs	ca. 15 min	

Patrick J. L. Taylor更為我們展示了許多用來篩選真菌毒素之方法，像是：

固相淨化管柱



試紙條檢測



免疫親和管柱

八、 真菌毒素檢測方法的驗證和認證(Method Validation and Accreditation)：主講人為 TUBITAK-ATAL 的 Hamide Z Senyuva。

主要介紹方法確效及實驗室認證，方法確效分為三階段(1) In-house laboratory method validation：為確認一個方法可以表現多好的第一階段，利用單一分析者、單一實驗室及相同的儀器設備(2) Peer-verified laboratory method validation：經由第二個或第三個實驗室來確認這個方法的可信度(3) Inter-laboratory method validation：經由最少八個實驗室來確認此方法（一般建議由十二個實驗室來確認）。方法確效的特性有特異性(Specificity)、最低偵測極限(Limit of Detection, LOD)、最低定量極限(Limit of Quantification, LOQ)、線性關係(Linearity)、準確性(Accuracy)、精密度(Precision)及重現性(Ruggedness)。

九、 真菌毒素檢測能力測試(Proficiency Testing)：主講人為英國中央科學實驗室(Central Science Laboratory)的 John Gilbert 。

介紹英國中央科學實驗室及其能力測試，及有關FAPAS (Food Analysis Performance Assessment Scheme)之運作模式。在CSL(Central Science Laboratory)之下有四種能力測試：(1) FAPAS®：食品化學分析；(2) FEPAS®：食品微生物；(3) LEAP：環境及飲用水分析；(4) GeMMA：基因改造食品分析。目前參加FAPAS的約有80個國家，超過1300個實驗室參與，每年約舉辦190個能力測試項目。其中，有關真菌毒素的能力測試項目有：

(1)核果類、乾無花果、動物飼料、香辛料及嬰兒食品中的黃麴毒素B_1, B_2, G_1和G_2；(2)液狀牛奶及奶粉中的黃麴毒素M_1；(3)穀類、咖啡、乾果、嬰兒食品及酒類中的赭麴毒素A；(4)玉米中的伏馬毒素 B_1和B_2；(5) 穀類和動物飼料中的Deoxynivalenol (DON)；(6)穀類和嬰兒食品中的Zeralenone：(7)燕麥中的T-2毒素(預計2007年1月實施)。

FAPAS的結果一般以z-score表示：

$$z = \frac{(x - \hat{X})}{\sigma_p}$$

x = participant's result

$\hat{X}$ = the assigned value

σ_p = target standard deviation

通常|z| ≦ 2，表示滿意；2＜|z| ≦ 3時，則為有疑慮的；|z| > 3表示不滿意。

十、 真菌毒素分析面臨的挑戰(Challenges for Mycotoxin Analysis - Multi-toxin LC/MS)：主講人為荷蘭食品安全研究所(RIKILT-Institute of Food Safety)的 Wim Traag。

主要介紹現今檢測真菌毒素所使用的儀器，個別優缺點及靈敏度比較。一般而言，以高效液相質譜儀(LC/MS)所需的分析時間最短，靈敏度最高。現今可利用 LC/MS 在 12 分鐘內同時檢測 17 種真菌毒素，不僅可以在短時間得到數據，且同時檢測多種真菌毒素，為快速有效率的檢測方法。

附圖一、SELAMAT 真菌毒素研習會講師群合影

附圖二、與參加 SELAMAT 真菌毒素研習會學員合影

第三天(12 月 12 日 星期二)

地點：北京「中國農業科學院植物保護研究所」(只有受邀會員國參加)

一、 取樣、樣品製備及分析物分離(Sampling, sample preparation and analyte isolation)：主講人為荷蘭公共衛生與環境國家研究院(RIVM)的 Hans van Egmond。

取樣、樣品製備及分析過程中的變數，分析物的分離及淨化。實驗過程中所造成的誤差，可能產生在取樣、樣品製備及分析三個過程中，其中以取樣過程中造成的誤差，影響實驗結果最為嚴重。最主要作為淨化步驟的為免疫親和管柱，使用免疫親和管柱優點為具選擇性，且可以將樣品濃縮，缺點為不適用於高濃度的有機溶液，容易受pH值影響，且價錢較貴。

二、 分析 Fuarium 毒素所需的儀器(Instrumental Techniques for Fusarium Toxin Analysis)：主講人為 TUBITAK-ATAL 的 Hamide Z Senyuva。

介紹分析 Fusarium 毒素所需的儀器，以及分析 Fusarium 毒素(Zearalenone、Fumonisin、Trichothecenes)之標準操作流程。

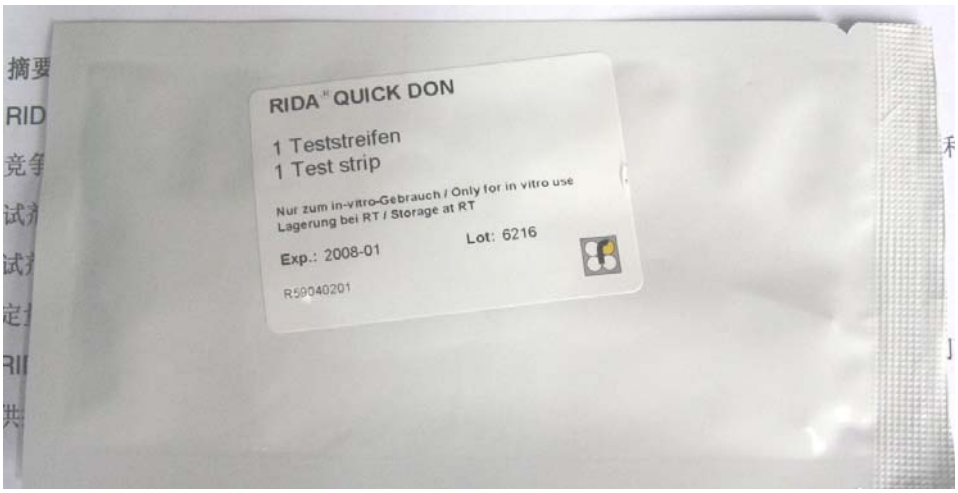
常用來分析 Fusarium 毒素的儀器如下表所示：

Technique	Derivatization	Mycotoxin
HPLC (FI)	OPA	Fumonisins
LC/MS	None	Fumonisins
HPLC (UV)	None	DON
GC&GC/MS	TFA,	DON, NIV, T2
LC/MS&TOF	None	Multi-toxin

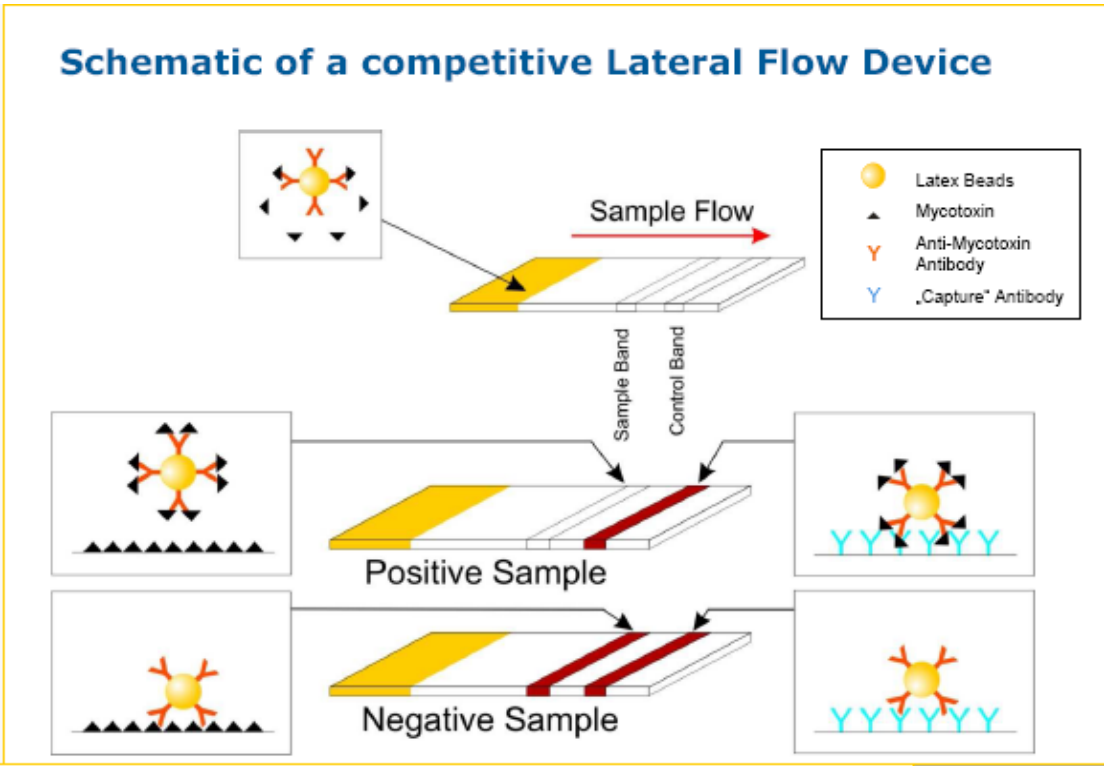
三、 RIDA® QUICK DON：為利用Lateral Flow Device原理的test strip，加入萃取液後，只需5分鐘即可知道檢體是否含有DON，可快速的定性檢測穀物中的DON。

原理：抗原抗體反應，針對DON的特異性抗體可與小麥樣品中的DON結合，結果可透過目測檢測線的顏色來判斷。質控線(C)不受樣品中是否含有DON的影響，質控線(C)清楚的出現，證明實驗是有效的。若觀察到檢測線(T)出現，則表示樣品中的DON含量小於或等於1 ppm；若不能觀察到檢測線(T)或很淡，則表示樣品中的DON大於或等於1 ppm，。

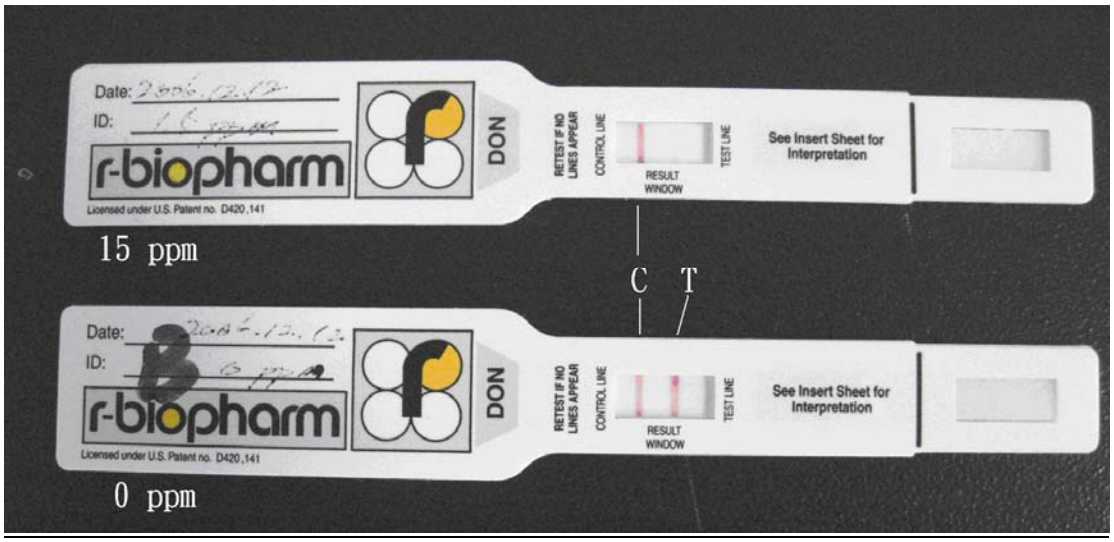
流程：取4 g樣品+20 mL PBS緩衝液（以1:5比例稀釋）→利用均質機萃取3分鐘→濾紙過濾→加入120 μL濾液於試條的檢測區→等待5分鐘→目測檢測線的顏色。



Lateral Flow Device的原理：



實驗結果：



陽性反應為只出現一條質控線（DON含量大於等於1 ppm），而陰性反應為出現質控線和檢測線共兩條線（DON含量小於1 ppm）。應盡量在5分鐘時進行評估，超過5分鐘後，兩條線都會有輕微變暗的現象，會影響檢測的結果。

四、 RIDASCREEN® FAST DON：使用競爭性酵素連結免疫吸附分析方法定量，穀類、麥芽及飼料中的DON。其中，小麥、大麥、大麥芽、燕麥和玉米的檢測方法是經過AOAC確效的方法。

規格：48孔盤－最多可檢測43個樣品

96孔盤－最多可檢測91個樣品

原理：抗原抗體反應。微孔盤上含有針對DON抗體的二次抗體。加入DON標準或樣品溶液、Enzyme Conjugate及抗體。游離的DON與Enzyme Conjugate競爭DON抗體，同時DON抗體與二次抗體連接，沒有連接的Enzyme Conjugate在洗滌的步驟中被除去，加入Substrate/Chromogen，結合的Enzyme Conjugate將紅色的Substrate/Chromogen轉化為藍色的產物。加入中止液後使顏色由藍色轉為黃色，在波長450 nm下測量，吸收強度與樣品中的濃度成反比。

流程：

(1) 取5 g已磨碎的樣品與100 mL蒸餾水混合。

(2) 以ULTRA-TURRAX®攪拌機萃取2分鐘。

(3) 以Whatman No.1濾紙過濾，得到樣品萃取液。

(4) 加入50 μL的標準品或樣品萃取液至個別的well(使用前所有試劑要先回復到室溫)。

(5) 加入50 μL Enzyme Conjugate至每一個well。

(6) 加入50 μL DON抗體溶液至每一個well，用手輕輕搖動微孔盤，使之混合，在室溫下反應5分鐘。

(7) 倒出well中的液體，將微孔盤倒置在吸水紙上拍打，以完全除去well中的液體，再各加入250 μL蒸餾水。重複上述步驟兩次以上。

(8) 加入100 μLSubstrate/Chromogen至每一個well，於室溫暗處下反

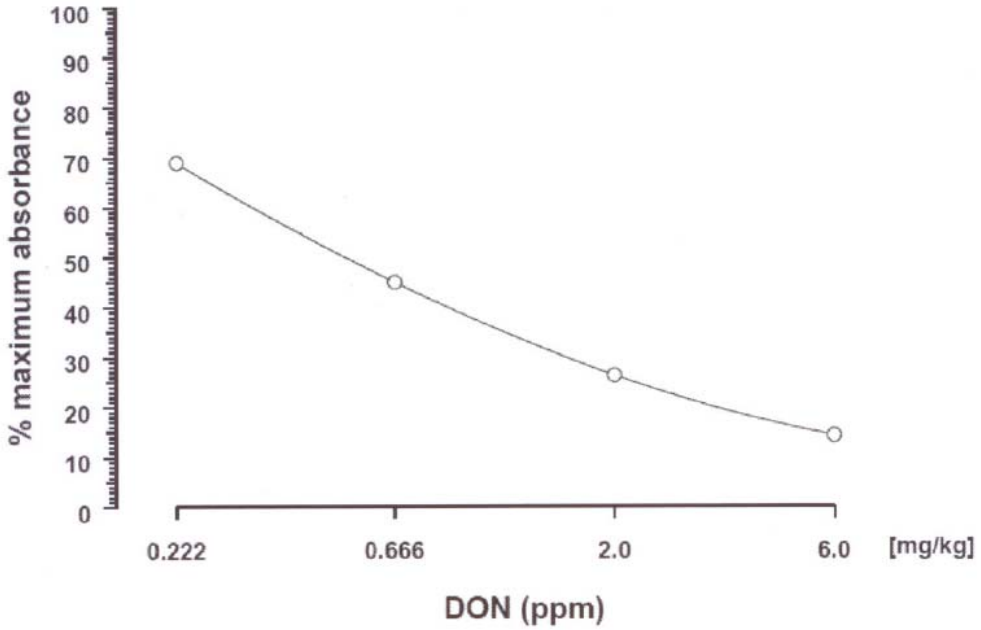
應3分鐘。

(9) 加入100 μL反應終止液至每一個well，用手輕輕搖動微孔盤，使之混合，在波長450 nm下測量吸光值（必須在加入終止液後10分鐘內讀取吸光值）。

ELISA test kit：

實驗結果：

得到DON標準曲線如下圖：



得到結果之數據，需利用R-Biopharm公司之軟體RIDA®SOFT Win計算，如果只做單一檢測，建議用logit/log方法計算；如果是雙重或多重檢測，

建議用cubic spline方法計算。

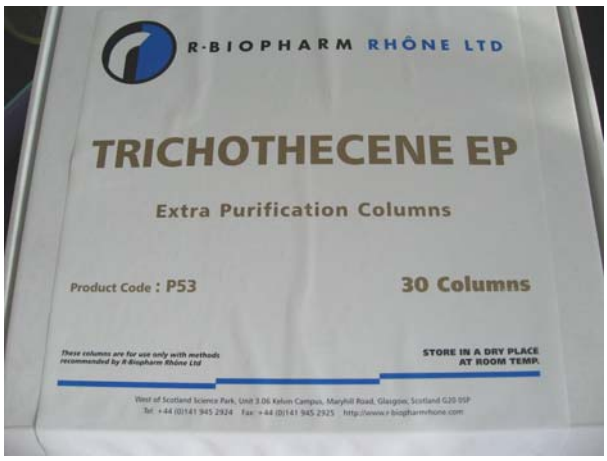
第四天(12月13日 星期三)

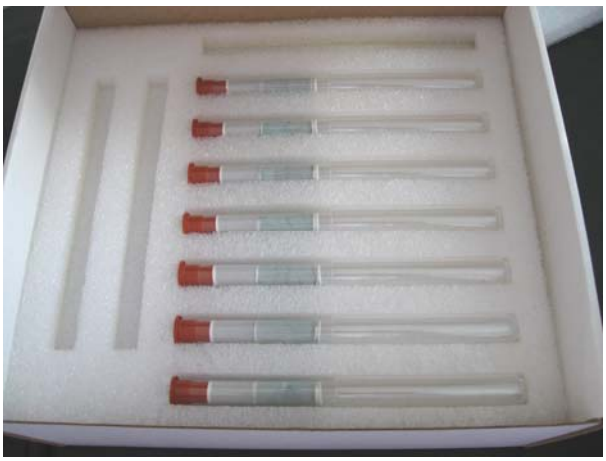
地點：北京「中國農業科學院植物保護研究所」(只有受邀會員國參加)

一、 觀看"分析 Fumonisins 操作方法"之影片，讓大家對實驗原理及步驟有初步了解。

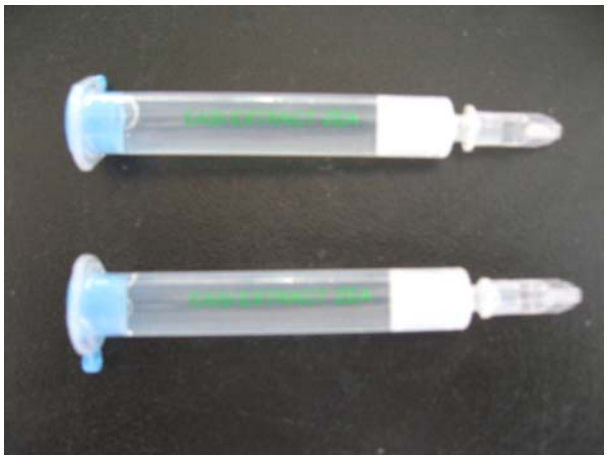
二、 實驗的部份，將學員分為三大組，檢測毒素為 trichothecens 及 zearalenone，使用高效液相層析質譜儀(LC/MS)、氣相層析質譜儀 GC/MS 及高效液相層析儀(HPLC)三種不同儀器分別進行檢測，最後為 Discussion Q&A。透過利用 immunoaffinity column 及 multifunctional column 進行樣品前處理之步驟，經過前處理後之樣品，即可以 LC/MS、GC/MS 及 HPLC 三種不同儀器分別進行檢測。HPLC 為目前最常被用來檢測真菌毒素的儀器，主要原因為分離效率高，分析速度快，且成本較低。GC/MS 主要檢測揮發性、且熱穩定性的樣品；LC/MS 主要檢測非揮發性、且熱不穩定性的樣品。

multifunctional column：

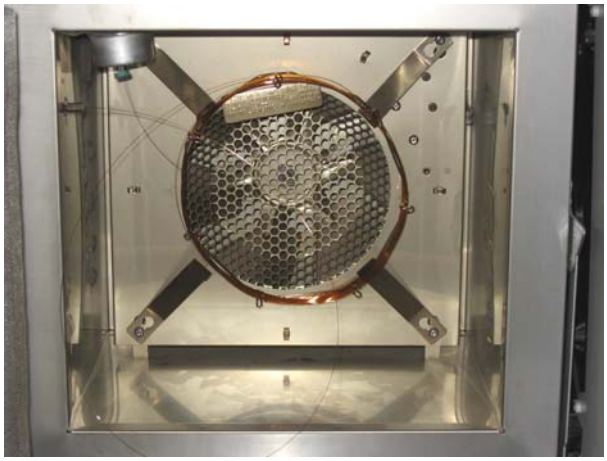




immunoaffinity column及自動快速濃縮儀：

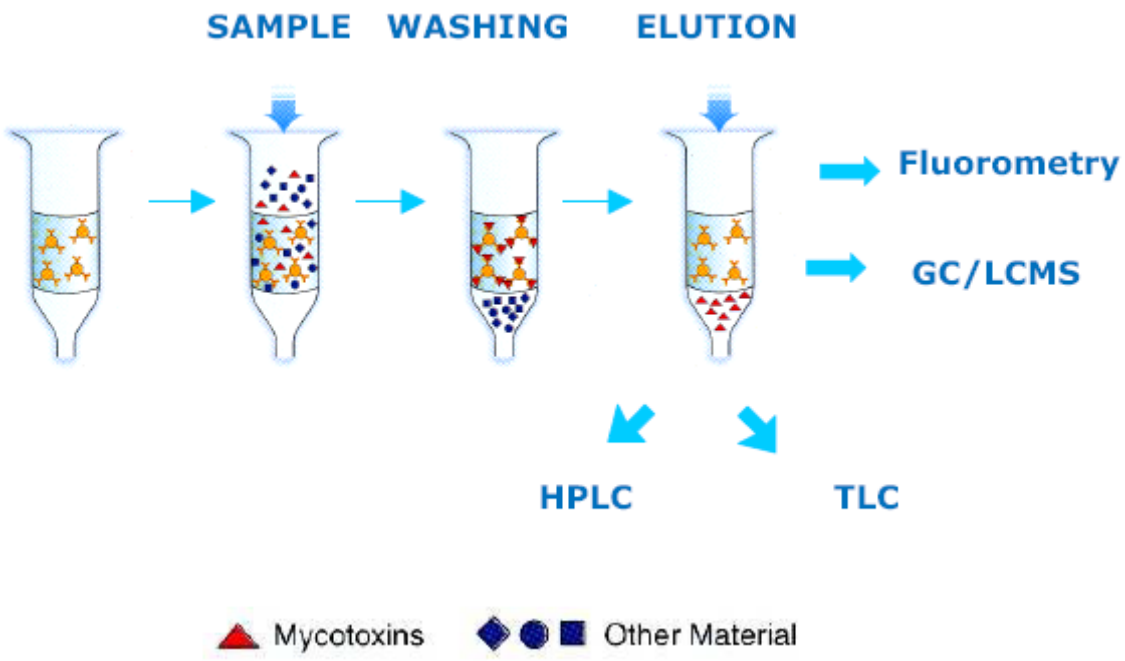


GC/MS

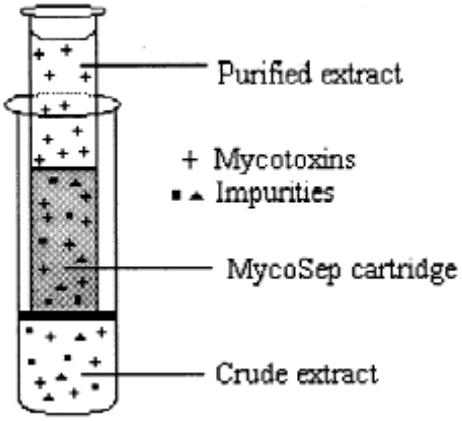


LC/MS

免疫親和管柱之原理：



固相淨化管柱之原理：



三、 最後為閉幕典禮，並且頒發與會人員證書。

於中國農業科學院植物保護所實驗室合影：

第五天(12月14日 星期四)

搭乘中國國際航空CA 117班機至香港機場，再搭乘國泰航空CX 408班機返回台灣。

參、心得與建議

(一) 目前FAPAS所舉辦有關真菌毒素的項目有(1)核果類、乾無花果、動物飼料、香辛料及嬰兒食品中的黃麴毒素B_1, B_2, G_1和G_2；(2)液狀牛奶及奶粉中的黃麴毒素M_1；(3)穀類、咖啡、乾果、嬰兒食品及酒類中的赭麴毒素A；(4)玉米中的伏馬毒素 B_1和B_2；(5) 穀類和動物飼料中的Deoxynivalenol；(6)穀類和嬰兒食品中的Zeralenone；(7)燕麥中的T-2毒素(預計2007年1月實施)。本局已連續多年參加FAPAS能力測試－花生粉中黃麴毒素，每年的實驗結果|z|-scores皆≦2，為肯定本局檢驗黃麴毒素的技術。日後更可以考慮參加其他真菌毒素的能力測試，藉以了解我國檢驗真菌毒素的能力是否達到國際水平。

(二) 目前本局針對真菌毒素已公告的檢驗方法有(1)花生、玉米、其他穀類及其製品中黃麴毒素B_1, B_2, G_1和G_2之檢驗；(2)液狀乳、粉狀乳及醱酵乳中黃麴毒素M_1之檢驗；(3)咖啡、酒類及葡萄汁、米麥製品中赭麴毒素A之檢驗；(4)玉米及其製品中伏馬毒素B_1及B_2之檢驗；(5)蘋果汁及蘋果泥中棒麴毒素之檢驗；(6)穀類及其製品中脫氧雪腐鐮刀菌烯醇(deoxynivalenol, DON)之檢驗。另外，進行中的實驗有：(1)玉米製品中T-2及HT-2毒素之檢驗；(2)穀類及紅麴製品中橘黴素(Citrinin)之檢驗；(3)穀類製品中雪腐鐮刀菌烯醇(Nivalenol, NIV)之檢驗；(4)穀類製品中玉米赤黴烯酮(Zeralenone)之檢驗。若與英國FAPAS能力測試項目相較，本局所建立的真菌毒素檢驗方法，不僅僅包含目前FAPAS組織能力測試項目的真菌毒素種類，同時也更積極的研究其他真菌毒素的檢驗方法。

(三) 本局除了公告之檢驗方法：以專一性較高之免疫親和管柱搭配HPLC進行實驗為主。而其它檢驗方式，如：固相淨化管柱、酵素連結

免疫吸附分析法及串聯式高效液相層析質譜儀(LC/MS/MS)，亦在本局之測試項目內。所以本次研習會所學習的儀器和設備，大多為之前研究過的檢驗方法，可知我們已與國際接軌，所使用的檢驗方法也都與國際上公認相符。

(四) SELMAT組織成立的宗旨即在科學的平台上互相交流，不介入政治議題，本次參與該研討會也備受禮遇，因此建議可多派員參加類似之會議，瞭解國際新知及互相交流討論，可提升本局專業識能。

(五) 藉由此次研習會，與各國專家學者建立順暢的資訊互惠交流管道，例如英國中央科學實驗室(Central Science Laboratory)的John Gilbert，荷蘭食品安全研究所(RIKILT-Institute of Food Safety)的Wim Traag、Hans Marvin及Piet Stouten、韓國之食品工業發展研究所(Korea Food Research Institute, KFRI)的Minseon Koo、新加坡農獸局(Agri-Food and Veterinary Authority, AVA)的李秀連小姐、日本食品安全總合研究所的久城真代小姐等，作為本局掌握國際資訊的橋樑，對於本局日後取得各國對於真菌毒素領域的資訊上有莫大的實質助益。

附件一、真菌毒素分析與風險評估研習會之介紹

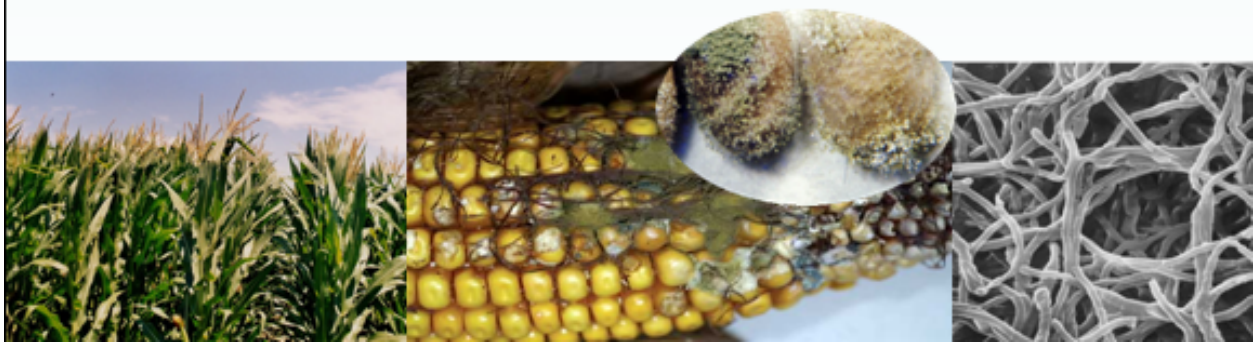
SELAMAT Workshop SELAMAT Workshop on Fusarium toxins - Fumonisins, DON, T-2 toxin and Zeralenone

11-13th December 2006
Beijing (China)

This event, which is organised and funded by SELAMAT and hosted by Chinese Academy of Agricultural Sciences (CAAS), will comprise a day of introduction to Fusarium toxins- Fumonisins, DON, NIV, T-2 toxin and Zeralenone testing for a general audience, including those responsible for managing contaminant monitoring programmes. This will be followed by two days of training with a more practical emphasis for a limited group of analysts which will involve laboratory demonstrations and hands-on activity.

http://www.selamat.net

Venue:- Institute of Plant Protection, CAAS,
2 West Yuanmingyuan Road, Haidian, Beijing



11th December– Overview of Fusarium Toxin analysis

- Welcome and Introduction to SELAMAT Project

H. Marvin (RIKILT)

- Recent developments in the risk assessment of Fusarium toxins

J Gilbert (CSL)

- Regulations and guidelines for Fusarium toxins in food and feed

H van Egmond (RIVM)

- Fusarium Toxin Analysis an overview

J Gilbert (CSL)

LUNCH

- Occurrence of Fusarium toxins in food and feed

H van Egmond (RIVM)

- Screening Methods for Fusarium toxins

P Taylor (RBR)

- Method Validation and Accreditation

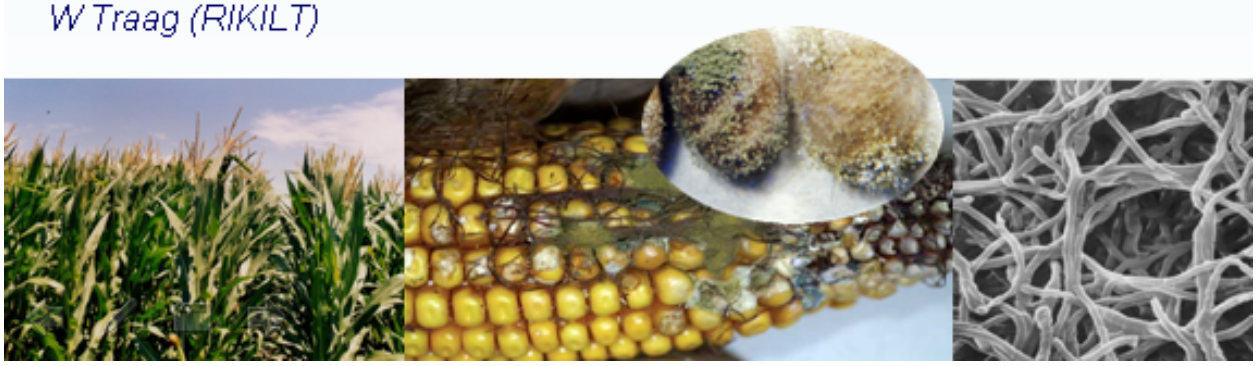
H Senyuva (TUBITAK-ATAL)

- Proficiency Testing

J Gilbert (CSL)

- Challenges for Mycotoxin Analysis - multi-toxin LC/MS

W Traag (RIKILT)



12th December– Lectures and Practical Demonstration

Restricted to 25 participants

- Basic principles Analytical Quality Assurance (inc standards)

W Traag (RIKILT)

- Sample preparation techniques (clean-up)

H van Egmond (RIVM)

- Instrumental Techniques for Fusarium Toxin Analysis

H Senyuva (TUBITAK-ATAL)

LUNCH

- Fusarium Toxin Screening Tests-

  Demonstration of and hands-on analysis using RIDASCREEN

P Taylor (RBR)

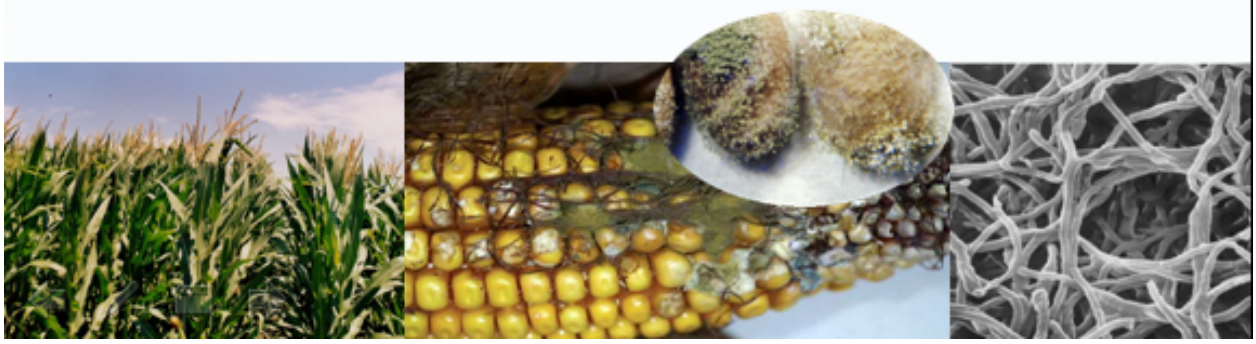
Fumonisin,

T-2 Toxin,

DON,

Zearalenone,

- Discussion Q&A



13th December– Lectures and Practical Demonstration

Restricted to 25 participants

- Group A Demonstration of DON Analysis Using IAC Clean-up and HPLC

W Traag (RIKILT)

- Group B Demonstration of Fumonisins B1 and B2 Analysis Using IAC Clean-up and HPLC with Pre-column Derivatization

Hans van Egmond (RIVM)

LUNCH

- Group A Demonstration of Trichothecenes Analysis Using Solid Phase Clean-up and GC/MS

J Gilbert (CSL)

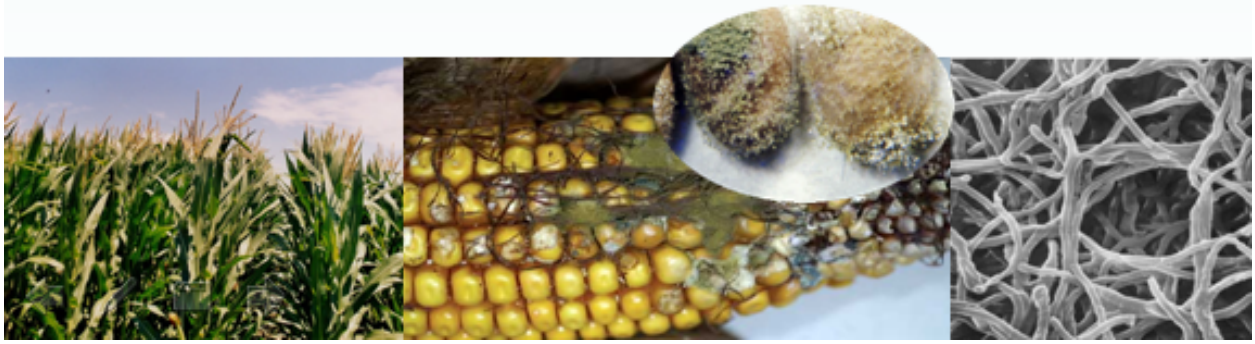
- Group B Demonstration of Zearalenone Analysis Using IAC Clean-up and HPLC

H Senyuva (TUBITAK-ATAL)

- Both Groups together - Challenges for Mycotoxin Analysis - multi-toxins LC/MS

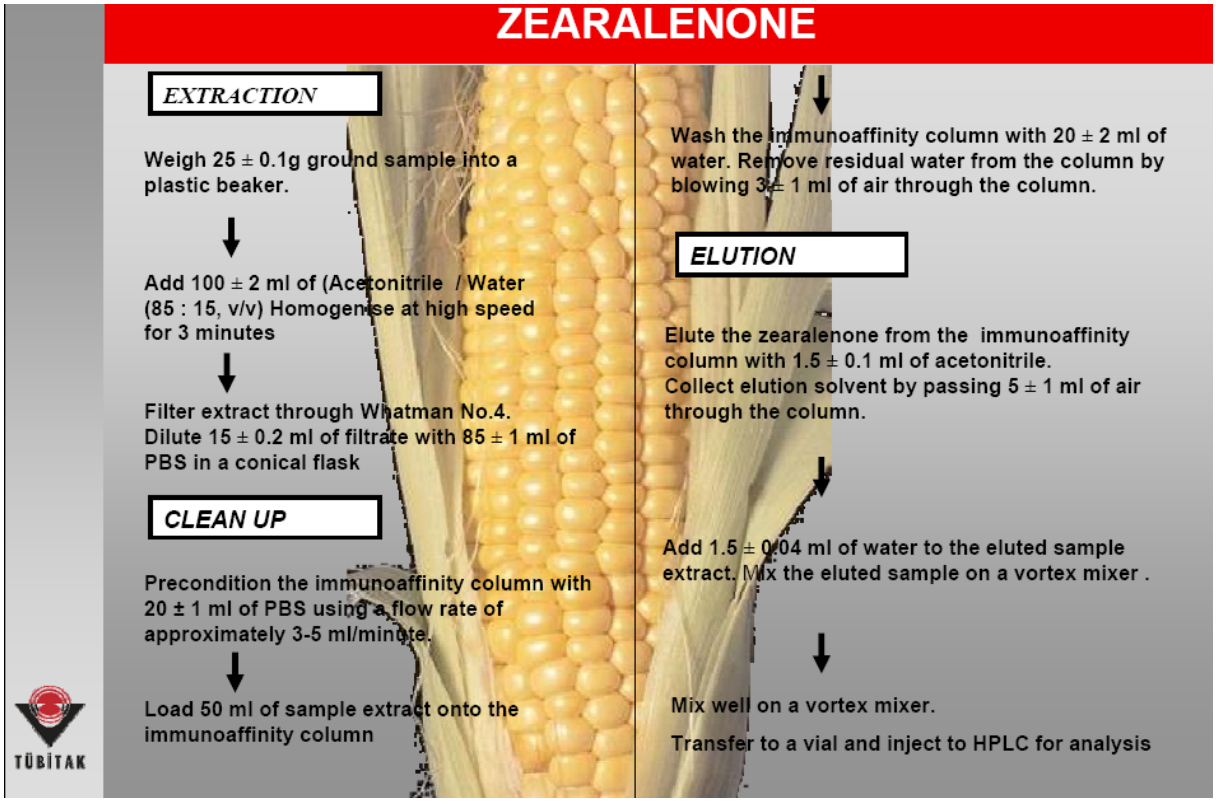
W Traag & H Senyuva

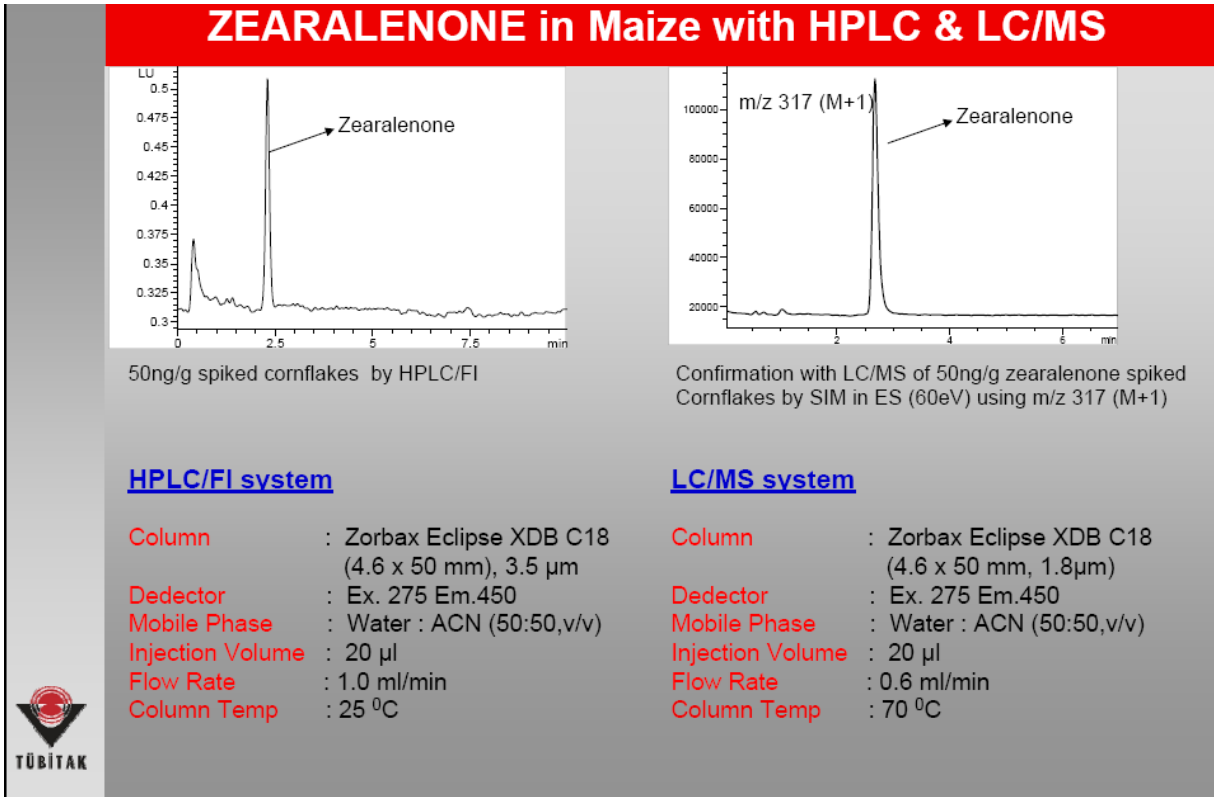
- Discussion Q&A



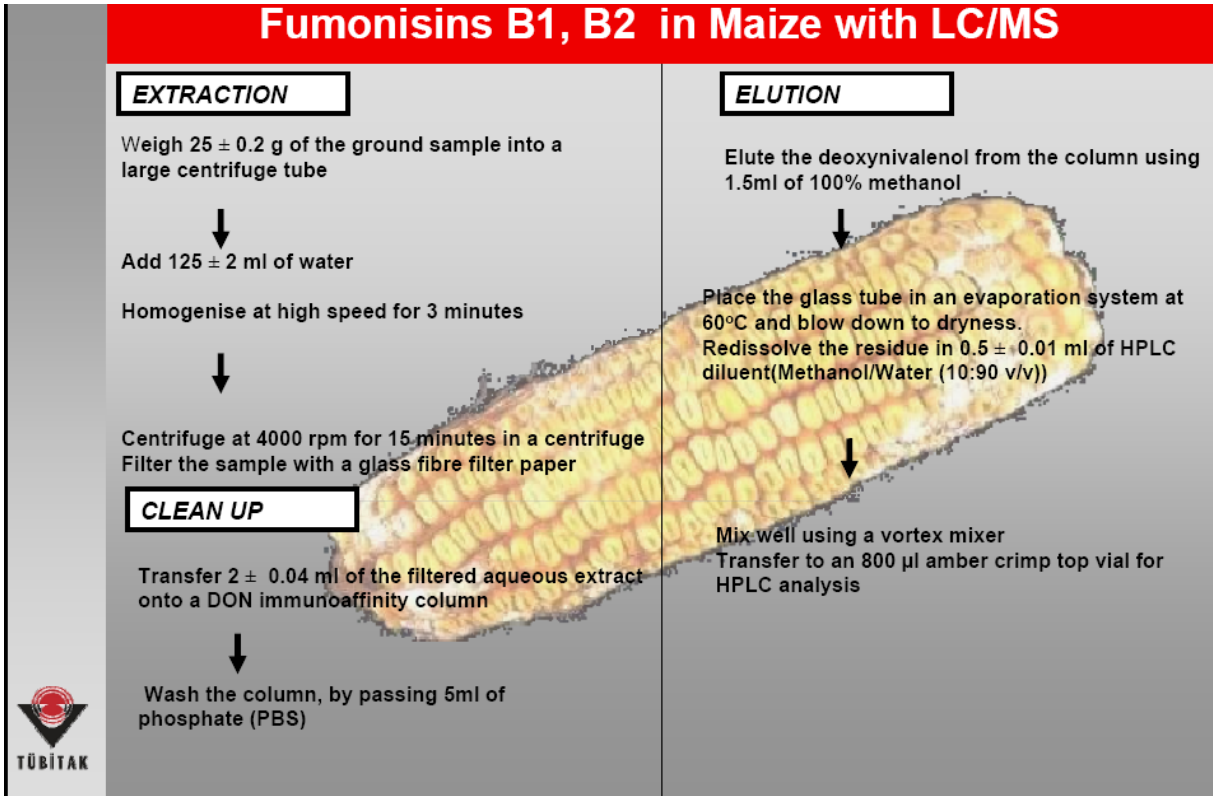
附件二、分析 Zearalenone、Fumonisins 及 Trichothecenes 之方法及條件

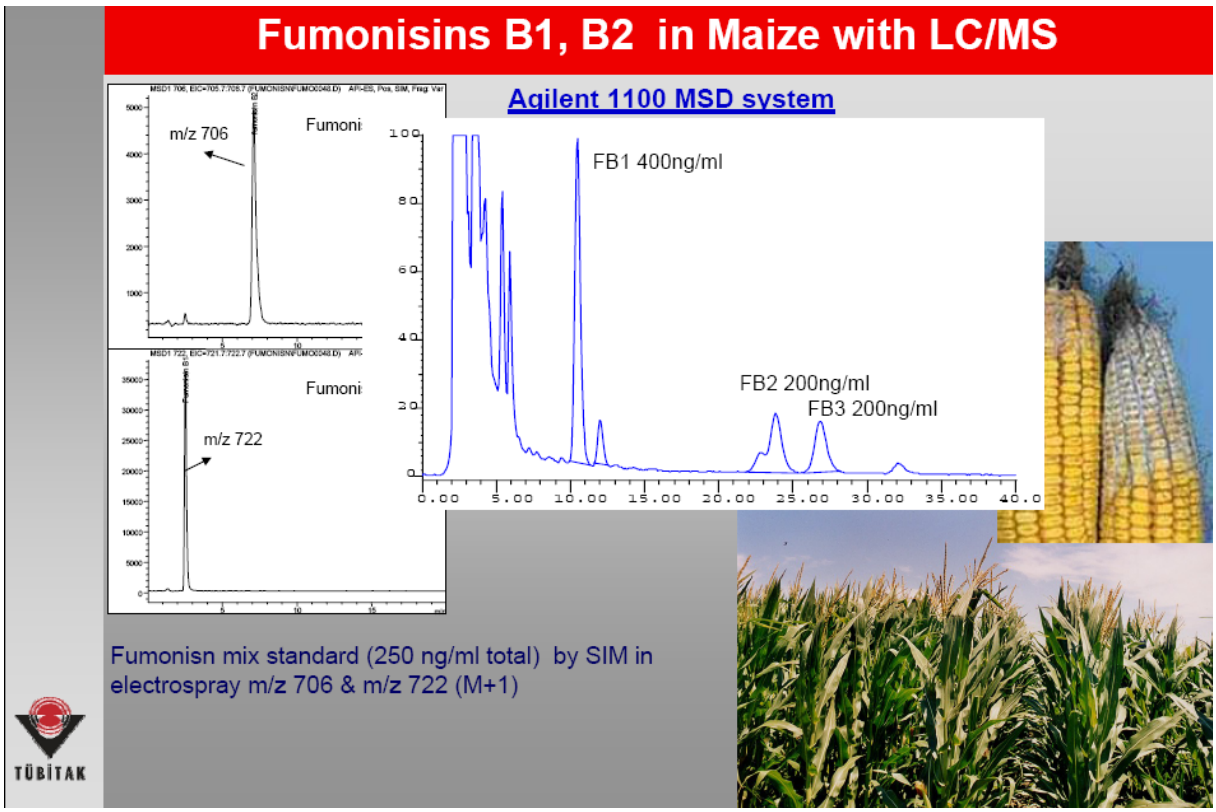
利用 HPLC 及 LC/MS 分析 Zearalenone 之方法及條件



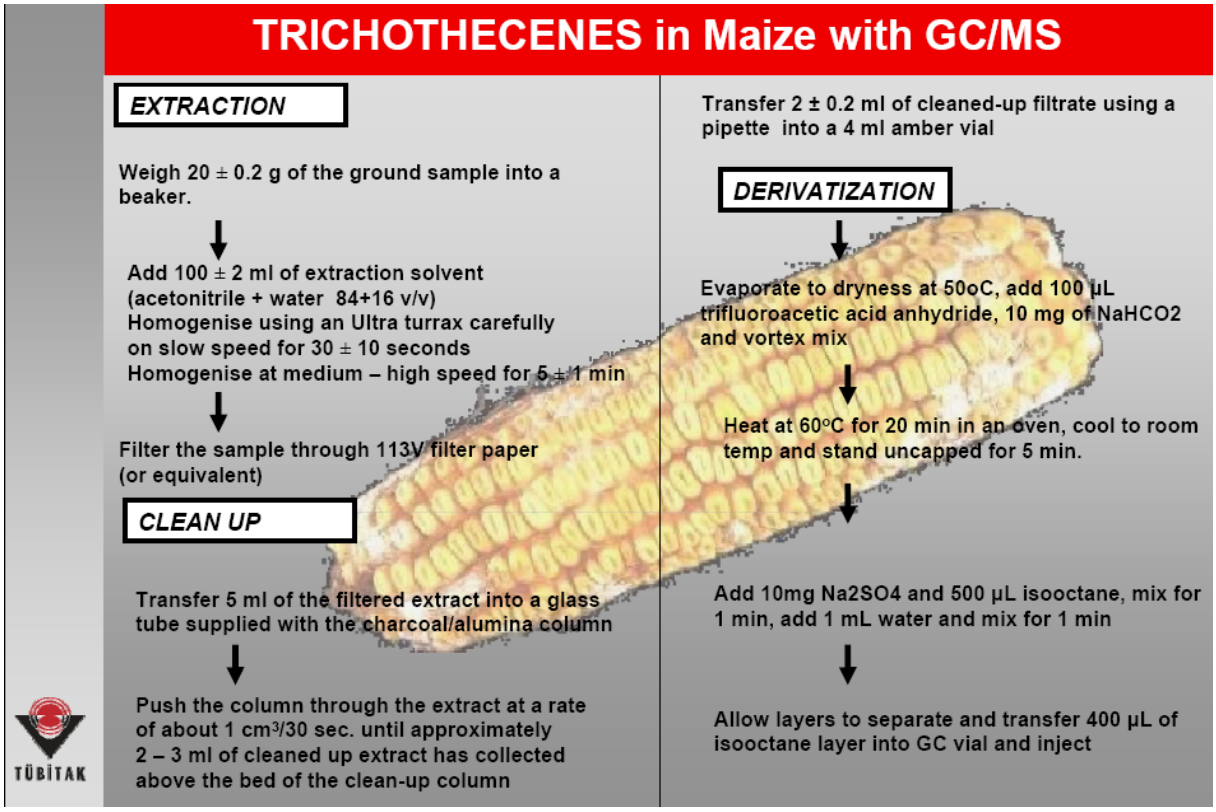


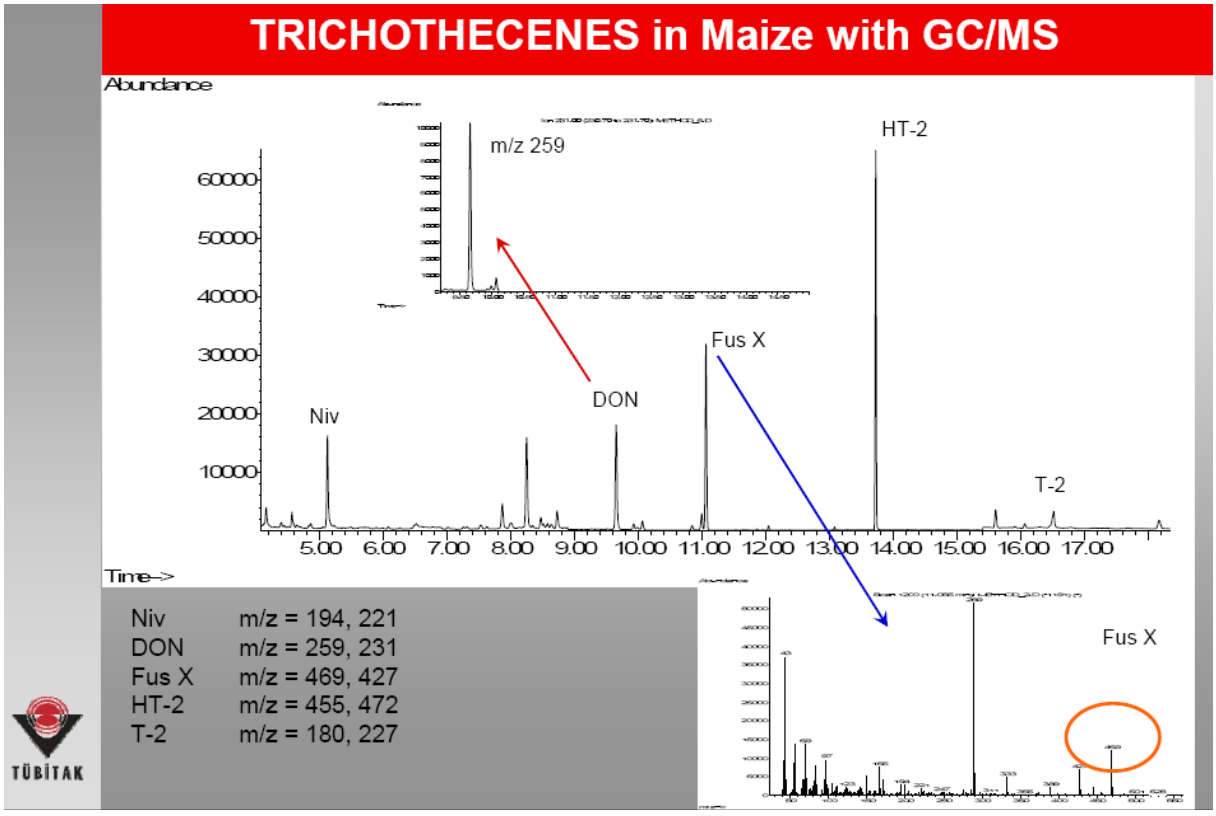
利用 LC/MS 分析玉米中 Fumonisins B_1 及 B_2 之方法及條件





利用 GC/MS 分析玉米中 Trichothecenes 之方法及條件





附件三、RIDASCREEN®FAST DON 操作流程

APPLICATION NOTE

CEREALS

RIDASCREEN® FAST DEOXYNIVALENOL

Detection of DEOXYNIVALENOL– ELISA SYSTEM

METHOD

Sample Preparation

a) Weigh 5 g of ground sample into a suitable container.

b) Add 25 ml of distilled water *

c) Shake vigorously for three minutes (manually or with shaker)

d) Filter the extract through Whatman No. 1 filter

e) Dilute the supernatant or filtrate 1:7 (1+6) with sample dilution buffer

f) Use 50 µl of the diluted supernatant or filtrate per well in the test

*) Sample size may be increased if required, but the volume of water must be adapted accordingly, e.g.: 25 g in 125 ml distilled water or 50 g in 250 ml distilled water

ELISA

a) Insert a sufficient number of microtiter wells into the microwell holder for all standards and samples to be run in duplicate. Record standard and sample positions.

b) Pipet 50 µl of standard or prepared sample to separate wells; use a new pipette tip for each standard or sample.

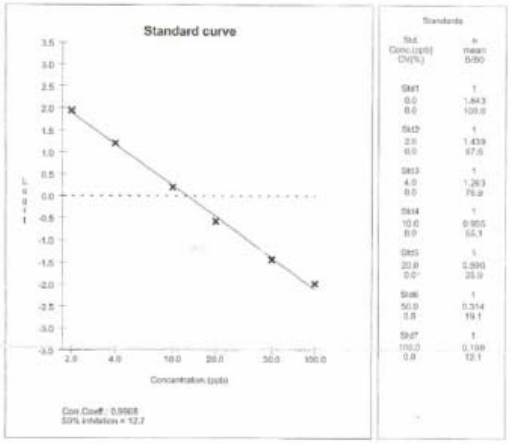
c) Add 50 µl of enzyme conjugate (red cap) to the bottom of each well.

d) Add 50 µl of the anti-deoxynivalenol antibody (black cap) to each well. Mix gently by rocking the plate manually and incubate for 30 min at room temperature (20 - 25 °C / 68 - 77 °F).

e) Dump the liquid out of the wells into a sink. Tap the microwell holder upside down onto a clean filter towel (three times in a row) to remove all remaining liquid from the wells. Using a multichannel pipette, fill the wells with 250 µl per well of washing buffer (10.3.). Empty the wells again and remove all remaining liquid. Repeat the washing step two more times.

f) Add 2 drops (alternatively 100 µl) of substrate/chromogen to each well (white dropper). Mix gently by rocking the plate manually and incubate for 15 min at room temperature (20 - 25 °C / 68 - 77 °F) in the dark.

g) Add 2 drops (alternatively 100 µl) of stop solution to each well (yellow dropper). Mix gently by rocking the plate manually and measure the absorbance at 450 nm against an air blank. Read within 10 minutes.



REFERENCES

1- RIDASCREEN DON – Enzyme immunoassay for the quantitative analysis of deoxynivalenol, Art No.: R5906

附件四、Trichothecenes 分析方法

APPLICATION NOTE

TRICHOTHECENES

EXTRACTION

Weigh 20 ± 0.2 g of the ground sample into a beaker.

↓

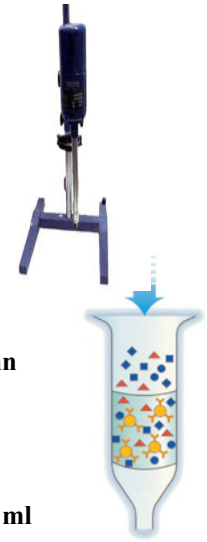
Add 100 ± 2 ml of extraction solvent (acetonitrile + water 84+16 v/v)

Homogenise using an Ultra turrax carefully on slow speed for 30 ± 10 seconds

Homogenise at medium – high speed for 5 ± 1 min

↓

Filter the sample through 113V filter paper (or equivalent)



CLEAN UP

Transfer 5 ml of the filtered extract into a glass tube supplied with the charcoal/alumina column

↓

Carefully insert the plunger of the clean-up column into the glass tube

Push the column through the extract at a rate of about 1 cm^3/30 sec. until approximately 2 – 3 ml of cleaned up extract has collected above the bed of the clean-up column

↓

Transfer 2 ± 0.2 ml of cleaned-up filtrate using a pipette into a 4 ml amber vial

DERIVATISATION

↓

Evaporate to dryness at 50°C, add 100 µL trifluoroacetic acid anhydride, 10 mg of $NaHCO_2$ and vortex mix

↓

Heat at 60°C for 20 min in an oven, cool to room temp and stand uncapped for 5 min.

↓

Add 10mg Na_2SO_4 and 500 µL isooctane, mix for 1 min, add 1 mL water and mix for 1 min

↓

Allow layers to separate and transfer 400 µL of isooctane layer into GC vial and inject

APPLICATION NOTE

TRICHOTHECENES

Application note for analysis of trichothecenes by using immunoaffinity clean-up and GC/MS analysis*

INTRODUCTION

T-2 and HT-2 toxins are type-A trichothecene mycotoxins, which have closely-related epoxy sesquiterpenoid structures. Surveys have shown the presence of T-2 and HT-2 toxins in grains such as wheat, maize, oats, barley, rice, beans, and soya beans as well as in some cereal-based products. T-2 and HT-2 toxins have been reported to be produced by *Fusarium sporotrichioides, F. poae, F. equiseti,* and *F. acuminatum*.

T-2 toxin is the trivial name for 4β,15-diacetoxy-3α,dihydroxy-8α-[3-methylbutyryl-oxy]-12,13-epoxytrichothec-9-ene, which is shown in Figure 1. Corresponding to the molecular formula $C_{24}H_{34}O_9$, its relative molecular mass is 466.5 g/mol. 15-Acetoxy-3α,4β-dihydroxy-8α-[3-methylbutyryloxy]-12,13-epoxytrichothec-9-ene is the systematic name of HT-2 toxin. The molecular formula is $C_{22}H_{32}O_8$, and the relative molecular mass is 424.5 g/mol. The structures of T-2 and HT-2 toxins differ only in the functional group at the C-4 position. As T-2 toxin is readily metabolized to HT-2 toxin, these two mycotoxins were evaluated together.

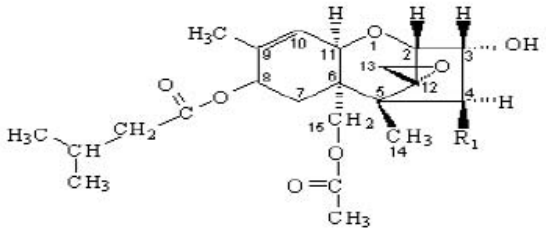


Figure 1 –Structure of type A trichothecenes : T-2 (R1:OAc) and HT-2 (R1:OH) toxins

* **Senyuva et al. 2006 unpublished method**

GC-MS EQUIPMENT

Column	HP-5MS, 30m x 0.25 mm x 0.25 µm or equivalent
Detector	Mass spectrometric (SIM)
Carrier gas	He (99.999%)
Flow rate	1.0 mL/min
Injector	1µl
Injector type	Splitless for 2 min

GC column temperature ramp

Time / min	Initial temp. / °C	Ramp / °C/min	Final temp / °C	Time held / min
0	120	-	120	2
2	120	30	175	3
6.8	175	10	220	0
11.3	220	25	270	5

Standard preparation

To prepare 100 µg/ml stock solution, dissolve the crystalline standard with 5ml acetonitrile.

GC Standards

Standard solutions are prepared using blank sample matrices. Following the procedures under the title of "derivatization", perform a single extraction of a matrix blank and clean-up more than 5 portions of blank sample extract. Transfer 2 ml aliquots of the cleaned-up extract to six individual 4 ml amber vials. Evaporate the cleaned-up filtrate under N2 (60^0C) and add the amount of mixed trichothecenes working solutions listed in Table to the dried blank extracts. Evaporate again to dryness and derivatise.

Derivatisation

Evaporate the cleaned-up filtrate to dryness using a heating block set at approximately 50°C (NB ensure the vial is absolutely dry before proceeding, if the vial is not dry the derivatisation step will not work properly). Add 100 µl of trifluoroacetic anhydride and approximately 10 mg sodium hydrogen carbonate and vortex mix. Heat at 60°C for 20 min in an oven. Afterwards, allow to cool to room temperature, and then place the uncapped vial for 5 min in a fume cupboard. Add approximately 10 mg anhydrous sodium sulfate and 500 µl of isooctane and mix using a vortex mixer for 1 min. Add 1 ml of water, and mix using a vortex mixer for 1 min. Allow the two layers to separate and transfer approximately 400 µl of the isooctane layer only to a GC vial for analysis (NB take care not to transfer any aqueous layer across to the GC vial).

Working Calibrant Solutions

Standards prepared in 5 ml tube.

Standard solution	Standard concentration (µg/ml)	Volume of mixed standard solution (µl)	Equivalent sample concentration (µg/kg)
1	0.8	40 (10 µg/ml)	1000
2	0.6	30 (10 µg/ml)	750
3	0.4	20 (10 µg/ml)	500
4	0.2	100 (1 µg/ml)	250
5	0.08	40 (1 µg/ml)	100
6	0.04	20 (1 µg/ml)	50

METHOD

a) Weigh 20 ± 0.2 g of the ground sample into a beaker.

b) Add 100 ± 2 ml of 84% acetonitrile.

c) Homogenise using an Ultra turrax carefully on slow speed for 30 ± 10 seconds to ensure the sample is mixed with the solvent.

d) Homogenise at medium – high speed for 5 ± 1 min.

e) Filter the sample through Whatman 113 filter paper (or equivalent).

f) Transfer 5 ml of the filtered extract into a glass tube supplied with the charcoal/alumina column

g) Carefully insert the plunger of the clean-up column into the glass tube.

h) Slowly push the column through the extract at a rate of about 1 cm^3 per 30 seconds until approximately 2 – 3 ml of cleaned up extract has collected above the bed of the clean-up column.

i) Transfer 2 ± 0.2 ml of cleaned-up filtrate using a pipette into a 4 ml amber vial.

j) Follow the derivatisation procedure.

Chromatograms

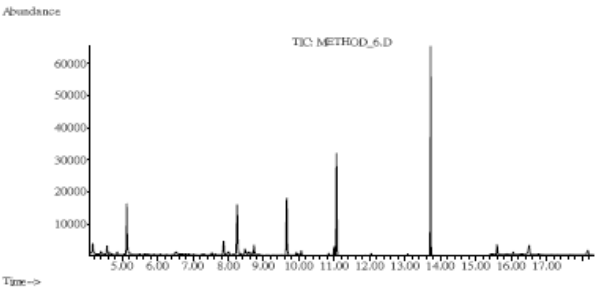


Figure 1. TIC of 1 µg/ml mixed standards with GC/MS detection

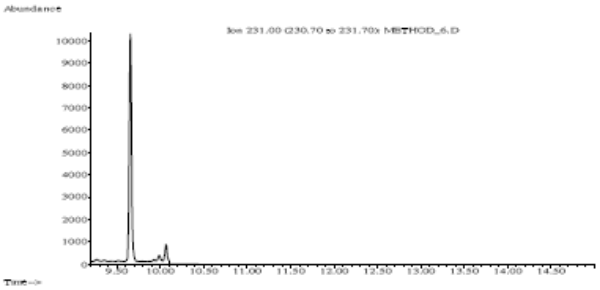
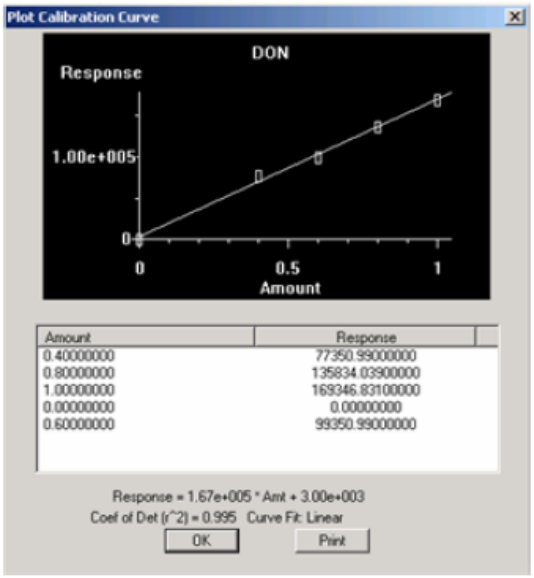


Figure 2. 1 µg/ml DON standard with GC/MS detection by SIM in EI mode (70eV) using m/z 231

CALIBRATION GRAPH (GC/MS)



附件五、Zearalenone 分析方法

APPLICATION NOTE

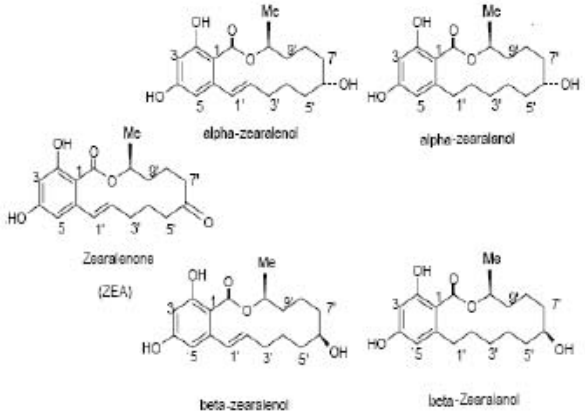
ZEARALENONE

Application note for analysis of zearalenone by using immunoaffinity clean-up and HPLC analysis*

INTRODUCTION

Zearalenone is a mycotoxin that can be produced by several field fungi including *Fusarium graminearum* (Gibberella zeae), *F. culmorum, F. cerealis, F. equiseti* and *F. semitectum*. Fungi of the genus *Fusarium* infect cereals pre-harvest in the field during blooming, but growth and toxin production may also occur post-harvest under poor storage conditions. The toxin is common in maize, but because the spores of Fusarium are ubiquitous, cereal crops such as barley, oats, wheat, rice, sorghum and soy beans are also susceptible to contamination with zearalenone, both in the temperate and warmer climate zones.

Zearalenone (formerly denoted F2-toxin) is a resorcyclic acid lactone chemically described as 6-(10-hydroxy-6-oxo-trans-1-undecenyl)-β-resorcyclic acid lactone ($C_{18}H_{22}O_5$, MW: 318.36) The structure of zearalenone and its major metabolites are shown in Figure below.



GENERAL HPLC EQUIPMENT

Analytical column	Zorbax Eclipse XDB C18 (50mm x 4.6 mm i.d.), 3.5 µm particle size (or equivalent).
Fluorescence Detector	Excitation = 275, Emission = 450 nm.
Column Heater	25°C
Injection volume	20 µl
Mobile Phase	Water / Acetonitrile mixture (50:50 v/v)
Flow Rate	1.0 ml/min

Stock Standard Solution

Ready to use zearalenone (1000 ng/ml) in acetonitrile can be used to prepare the following working standards.

Store stock standard solution in -20 °C)

Dilution Solvent

Prepare solvent for dilution calibrant solution, acetonitrile:water (50:50, v/v).

* Senyuva et al. 2006 unpublished method

Working Calibrant Solutions

Working Standard	Working Solution to use (volume,µl)	Dilution solvent (volume,µL)	Final Standart Concentration (ng/ mL)
1	50	4950	10
2	100	4900	20
3	150	4850	30
4	250	4750	50
5	375	4625	75
6	500	4500	100

Standards prepared in 5ml calibrated volumetric flasks.

METHOD

a) Weigh 25 ± 0.1g ground sample into a plastic beaker.

b) Add 100 ± 2 ml of extraction solution A (Acetonitrile / Water (85 : 15, v/v)).

c) Homogenise for 3 ± 1 min with an Ultra Turrax set at high speed.

d) Filter extract through Whatman No.4 or Whatman 113V (fluted filter)

e) Dilute 15 ± 0.2 ml of filtrate with 85 ± 1 ml of PBS in a conical flask.

f) Pre-condition the immunoaffinity column with 20 ± 1 ml of PBS using a flow rate of approximately 3-5 ml/min.

g) Load 50 ml of sample extract onto the immunoaffinity at a speed of no more than 3 ml/min.

h) Wash the immunoaffinity column with 20 ± 2 ml of water.

i) Remove residual water from the column by blowing 3 ± 1 ml of air through the column.

j) Replace the waste collection beaker with a glass vial.

k) Slowly (at a speed of approximately 1-2 drops per second) elute the zearalenone from the immunoaffinity column with 1.5 ± 0.1 ml of acetonitrile. This can be carried out by gravity.

l) Ensure that all the elution solvent has been collected by passing 5 ± 1 ml of air through the column.

m) Mix the eluted sample on a vortex mixer.

n) Add 1.5 ± 0.04 ml of water to the eluted sample extract.

o) Mix well on a vortex mixer.

p) Transfer mixed sample extract to HPLC vial and inject 20µl amount onto HPLC for analysis.

CHROMATOGRAMS

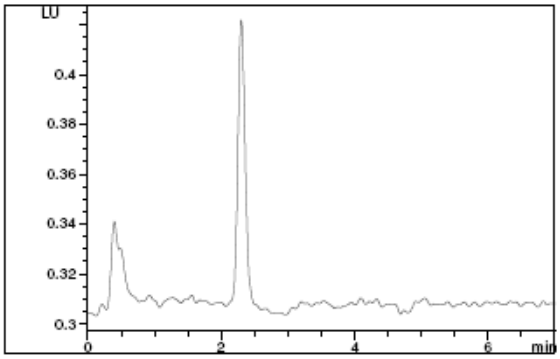


Figure 1. 25ng/ml zearalenone standard by HPLC with fluorescence detection

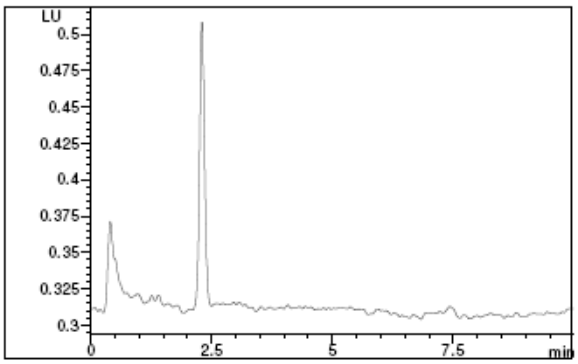


Figure 2. 50ng/g spiked cornflakes by HPLC with fluorescence detection

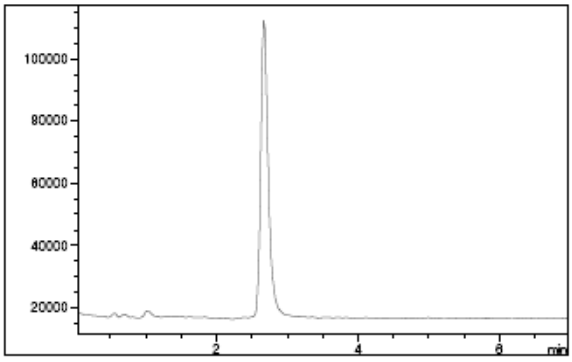
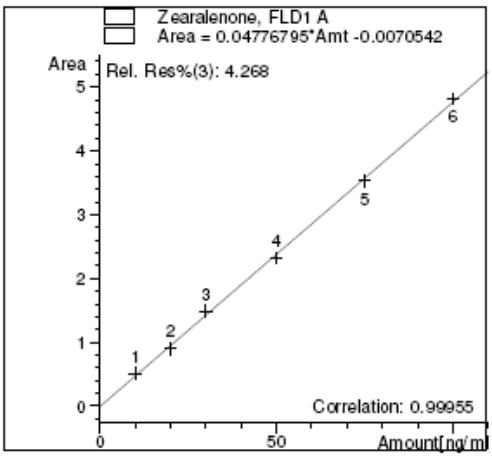


Figure 3. Confirmation with LC/MS of 50ng/ml zearalenone spiked cornflakes by SIM in electrospray (60eV) using m/z 317 (M+1)

HPLC conditions:
Column: Zorbax Eclipse XDB C18 (50mm x 4.6, 1.8µm)
Mobile Phase : 50% ACN + 50% Water
Flow rate : 0.6 ml/min
Column heater: 70^{0}C

CALIBRATION GRAPH (HPLC/FLD)



BACKGROUND LITERATURE

Senyuva et al. 2006 unpublished method

Fazekas B, Tar A. Determination of zearalenone content in cereals and feedstuffs by immunoaffinity column coupled with liquid chromatography. *J AOAC Int.* 2001 **84**:1453-9

Visconti, A., Pascale, M., Determination of zearalenone in corn by means of immunoaffinity clean-up and high-performance liquid chromatography with fluorescence detection *Journal of Chromatography A*, **815**:133-140