行政院國家科學委員會專題研究計畫　成果報告

食品中赭麴毒素之檢測分析及其致毒機制之探討

計畫類別：個別型計畫
計畫編號：NSC93-2313-B-040-004-
執行期間：93 年 08 月 01 日至 94 年 07 月 31 日
執行單位：中山醫學大學生物醫學科學學系

計畫主持人：余豐益

計畫參與人員：杞純鳳

報告類型：精簡報告

處理方式：本計畫涉及專利或其他智慧財產權，1 年後可公開查詢

中　華　民　國 94 年 10 月 31 日

行政院國家科學委員會補助專題研究計畫 ■ 成 果 報 告 □期中進度報告

食品中赭麴毒素之檢測分析及其致毒機制之探討

計畫類別：■ 個別型計畫　　□ 整合型計畫
計畫編號：NSC　93－2313－B－040－004－
執行期間：　　93 年　8 月　1 日至　94　年 7　月 31　日

計畫主持人：余豐益
共同主持人：
計畫參與人員： 杞純鳳

成果報告類型(依經費核定清單規定繳交)：■精簡報告　□完整報告

本成果報告包括以下應繳交之附件：
□赴國外出差或研習心得報告一份
□赴大陸地區出差或研習心得報告一份
□出席國際學術會議心得報告及發表之論文各一份
□國際合作研究計畫國外研究報告書一份

處理方式：除產學合作研究計畫、提升產業技術及人才培育研究計畫、列管計畫及下列情形者外，得立即公開查詢
□涉及專利或其他智慧財產權，□一年□二年後可公開查詢

執行單位：中山醫學大學生命科學系

中　華　民　國　九十三　年　十　月　三十　日

附件二

可供推廣之研發成果資料表

□ 可申請專利　■ 可技術移轉　日期：94 年 10 月 31 日

國科會補助計畫	計畫名稱：食品中赭麴毒素之檢測分析及其致毒機制之探討 計畫主持人：余豐益 計畫編號：NSC 93-2313-B-040-004-　　學門領域：食品
技術/創作名稱	赭麴毒素檢驗試劑之開發
發明人/創作人	余豐益
技術說明	中文： 利用本計畫生產出針對赭麴毒素具有高度專一性之抗體, 利用一抗體開發出赭麴毒素直接競爭型酵素免疫分析法(direct competitive enzyme-linked immunosorbent assay, dc-ELISA)來分析食品或穀物中赭麴毒素的含量。
	英文：Polyclonal antibodies for ochratoxin A (OTA) were generated from rabbits after immunizing the animals with OTA conjugated with γ-globulin. Using these antibodies, a competitive direct enzyme-linked immunosorbent assay were used for the characterization of the antibodies and for analysis of the toxin in different food samples.
可利用之產業及可開發之產品	赭麴毒素快速檢驗試劑
技術特點	利用抗體之高度專一性開發出敏感度好, 速度快之赭麴毒素快速檢驗試劑
推廣及運用的價值	

※ 1. 每項研發成果請填寫一式二份，一份隨成果報告送繳本會，一份送　貴單位研發成果推廣單位（如技術移轉中心）。

※ **2. 本項研發成果若尚未申請專利，請勿揭露可申請專利之主要內容。**

※ 3. 本表若不敷使用，請自行影印使用。

行政院國家科學委員會專題研究計畫成果報告

食品中赭麴毒素之檢測分析及其致毒機制之探討

Analysis of ochratoxins in food and studies on its toxicological mechanism

計畫編號：NSC 93-2313-B-040-004

執行期限：93 年 8 月 1 日至 94 年 7 月 31 日

主持人：余豐益　　中山醫學大學生命科學系

一、中文摘要

赭麴毒素 A(Ochratoxin A, OTA)的多株抗體由赭麴毒素 A 與載體蛋白質接合物(OTA-γ globulin),免疫到兔子體內，接著收集純化免疫血清來得到辨識赭麴毒素的抗體，直接與非直接競爭型酵素免疫分析法(Competitive direct and indirect enzyme-linked imunosorbent assay)被用來測定抗體的特性和分析赭麴毒素在遭受污染食品中的含量。此一直接競爭型酵素免疫分析法對赭麴毒素 A 與其類似物赭麴毒素 B 和 C 有弱交叉反應,50%抑制濃度分別為 0.9, 110 和 0.54ng/mL。將黃豆樣品加入 10 to 250 ng/g 不同濃度之赭麴毒素以 50%甲醇水溶液萃取後以直接競爭型酵素免疫分析法分析回收率可達 85.9%。目前利用此一酵素免疫分析法分析 20 種不同的農產品中,其中有 12 個樣品遭受到 16 到 160 ng/mL 之污染。樣品中的赭麴毒素亦經由高效液相層析法(High performance liquid chromatography)來加以分析確認。

關鍵詞：赭麴毒素、酵素免疫分析法、高效液相層析法

Abstract

Polyclonal antibodies for ochratoxin A (OTA)were generated from rabbits after the animals had been immunized with either OTA-γ-globulin or OTA- keyhole limpet hemocyanin (KLH). A competitive direct enzyme-linked immunosorbent assay (cdELISA) and a competitive indirect ELISA (ciELISA) were used for the characterization of the antibodies and for analysis of OTA in various agricultural commodities. The antibody titers in the serum of rabbits immunized with OTA-γ-globulin were considerably higher than those in rabbits immunized with OTA-KLH. The antibodies from the rabbits immunized with OTA-γ-globulin were further characterized. In the cdELISA, the concentrations causing 50% inhibition (IC_{50}) of binding of OTA-horseradish peroxidase to the antibodies by OTA, ochratoxin B (OTB), and ochratoxin C (OTC) were found to be 0.90, 110 and 0.54 ng/mL, respectively. When 10 to 250 ng/g of standard OTA was spiked to soybean samples and then extracted with 50% aqueous methanol, the recovery rate of OTA was found to be 85.9% in the cdELISA . Analysis of OTA in various agricultural commodities showed that 12 of the 20 examined samples were contaminated with OTA at levels from 16 to 160 ng/g. The efficacy of cdELISA was also confirmed by the high-performance liquid

二、緣由與目的

Ochratoxin A (OTA)**(Fig.1.)** is a naturally occurring toxic metabolite produced primarily by *Aspergillus ochraceus* and *Penicillium verrucosum*. It has been found as a common contaminant of cereals or in other products like coffee beans, nuts, wine, and animal organs (*1-3*). Numerous studies have revealed the role of OTA as a major causative factor in mycotoxic porcine nephropathy in many European countries (*4, 5*). Toxicological studies indicate that OTA is a teratogenic, mutagenic and carcinogenic mycotoxin, which is generally absorbed from the gastrointestinal tract in animals and has

strong toxic effects on their livers and kidneys. (*1, 2, 4-7*). Although acute renal failure due to inhalation of OTA in human has been rarely reported (*8*), OTA, with a long half-life of 840 h in human blood, is frequently found at high levels in serum samples obtained from people living in regions where Balkan Endemic Nephropathy occurs. OTA is also associated with an increased incidence of tumors of the upper urinary tract in human (*9-11*). The International Agency for Research on Cancer (IARC) has classified OTA as a possible human carcinogen (group 2B). The European Union has also enacted a regulatory limit for the levels of OTA in cereals (5 μg/kg), roasted coffee (5 μg/kg) and instant coffee (10 μg/kg) (*12,13*).

To help minimize the risk of human and animal exposure to OTA, extensive research has been conducted to develop sensitive and specific methods for detection of OTA in food and feed samples. High-performance liquid chromatography (HPLC) fluorescence detection with good accuracy and reproducibility is most widely employed for monitoring OTA (*14-18)*. However, HPLC methods require highly qualified personnel and extensive sample cleanup as well as expensive equipment. Development of immunochemical approaches has led to more simple and rapid methods to monitor and quantify OTA in contaminated food and feed. Although several groups have developed immunoassays for OTA detection (*19-24*), most of the assays rely on the competitive indirect ELISA (ciELISA) in which OTA-protein conjugates were coated onto microplates as solid phase. Compared with the competitive direct ELISA (cdELISA), the ciELISA is more time-consuming. To effectively analyze OTA levels in various agricultural commodities, in the present study a new method for the production of polyclonal antibodies against OTA was developed, and a sensitive cdELISA was also established.

三、Results

Production of Polyclonal Antibodies. Sera collected from rabbits immunized with OTA-γ-globulin or with OTA-KLH were subjected to the indirect ELISA (iELISA). Typical titration curves of antibody titers obtained from an OTA-γ-globulin immunized rabbit over a period of 15 weeks are shown in **Figure 2.** Antibodies against OTA were detected in the sera of rabbits as early as 6 weeks after initial immunization. The antibody titer increased progressively with time and the highest titer was found in the sera of rabbits at the 15th week after two subsequent immunizations. The antibody titers of the rabbits immunized with OTA-KLH were found to be considerably lower than those of the rabbits immunized with OTA-γ-globulin. much less for the antiserum obtained from OTA-γ-globulin-immunized rabbits (1:1000 dilution) than those from OTA-KLH-immunized ones (1:500 dilution), the 15th week antiserum from rabbits immunized with OTA-γ-globulin was used in the subsequent studies. In the cdELISA, the concentrations causing 50% inhibition (IC_{50}) of binding of OTA-HRP with the antibodies by OTA, OTB and OTC were found to be 0.90, 110, and 0.54 ng/mL, respectively **(Figure 3)**. The relative cross-reactivities of the antibodies to OTB and OTC were calculated to be 0.8 and 167, respectively. Similar results were also obtained in the ciELISA, in which OTA-OVA was coated on the wells of ELISA plates to serve as solid-phase antigen. The concentrations causing 50% inhibition of binding of antibodies to the solid-phase OTA-OVA by free OTA, OTB, and OTC were found to be 10, 168, and 5 ng/mL, respectively **(Figure 4)**. The relative cross-reactivities of the antibodies to OTB and OTC in the ciELISA were calculated to be 6.0 and 200, respectively. Citrinin and L-phenylalanine, two molecules with chemical structures similar to a part of OTA molecule, at a concentration of 100 μg/mL, did not inhibit the binding of marker antigens with the antibodies in either ELISA system (data not shown).

Recovery of OTA Added to Soybean by cdELISA.

To investigate the recovery rate of OTA in cdELISA, OTA were first spiked into soybean samples at concentrations from 10 to 250 ng/g and then the samples were extracted with 50% aqueous methanol for recovery analysis. As shown in **Table 1**, the recovery rate for 10-250 ng/g of spiked OTA was ranged from 79.2 to 95% and the overall average was calculated to be 85.9% (CV, 6.5%).

Analysis of OTA in Various Agricultural Commodities with cdELISA.

Twenty samples were collected from local food stores and subjected to cdELISA for OTA determination; the results are presented in **Table 1.** Twelve of the 20 examined samples were found to be OTA or OTA analogs positive with levels ranged from 16 to 160 ng/g. Among eight examined corn samples, sample 1 had the highest level of OTA at 63 ng/g, but OTA in samples 3, 4 and 8 were below the detection limit. Buckwheat sample 10 was found to have the highest level of OTA at 144 ng/g among four selected cereal samples. In addition, bean samples 13 and 16 had OTA levels at 74 and 43 ng/g, respectively. One of three examined coffee samples contained 160 ng/g of OTA, but the other two were free of OTA.

HPLC Analysis of OTA.

To test the efficacy of the cdELISA for OTA detection in various agricultural samples, the OTA standard and selected ELISA-positive samples were further analyzed with a HPLC method. HPLC chromatograms for OTA standard (5 ng), buckwheat sample 10 and roasted coffee sample 20 are shown in **Figure 5**. OTA standard was well identified with a retention time of 8.3 min under the isocratic elution (**Figure 5A**). The extracts of sample 10 and sample 20 also showed an OTA peak with a retention time of 8.3 min (**Figure 5B**), and the peak areas on the basis of calibration curve were calculated to be 60 and 220 ng/g, respectively. The levels of other examined samples were below the HPLC detection limit, which was around 0.01 ng of OTA per injection (**Table 2**).

Chemical Confirmation of the Presence of OTA in Samples by HPLC

To further assure the presence of OTA in samples by HPLC, chemical derivatization of OTA into its methyl ester form was conducted with the boron trifluoride method (*32, 33*). After derivatization and HPLC analysis, the peak of standard OTA decreased and the peak representing methyl ester form of OTA appeared with a retention time of 18.5 min (Figure 6A). The same patterns were observed in the derivatized coffee sample 20 (Figure 6B) and buckwheat sample 10 (data not shown). These results identified the existence of OTA in coffee sample 20 and buckwheat sample 10.

四、計畫成果自評

四、計畫成果自評

本研究的主要目的是針對常污染食品之赭麴毒素生產抗體並且與建立一套酵素免疫分析法來快速檢測分析食品及及其致毒機制之探討，由於本計畫所生產之赭麴毒素抗體具有很好的專一性，因此建立之直接競爭酵素免疫分析法可配合實驗試已經所建立之高效液相層析法來分析食品中以及咖啡中此一毒素之分布。利用此一直接競爭酵素免疫分析法方法分析食品中赭麴毒素，不僅分析樣品可不經前處理而且可在短時間內分析大量樣品。目前此一研究報告已發表於 2005 年 Journal of Agricultural and Food Chemistry 53:6947-6953.

五、參考文獻

(1) IARC. Monographs on the evaluation of the carcinogenic risk of chemicals to human, Some naturally occurring substances: Food items and constituents, heterocyclic aromatic amines and mycotoxins. International Agency for Research on Cancer, 1993; Vol. 56. 489-521.

(2) Kuiper, G. T.; Scott, P. M. Risk assessment of the mycotoxin ochratoxin A. *Biomed. Environ. Sci.* **1989,** *2,* 179-248.

(3) Studer-Rohr, J.; Dietrich, D. R.; Schlatter, J.; Schlatter, C. The occurrence of

ochratoxin A in coffee. *Food. Chem. Toxicol.* **1995,** *33,* 341-345.

(4) Krogh, P.; Elling, F.; Friis, C.; Hald, B.; Larsen, A. E.; Lillehoj, E. B.; Madsen, A.; Mortensen, H. P.; Rasmussen, F.; Ravnskov, U. Porcine nephropathy induced by long -term ingestion of ochratoxin A. *Vet. Pathol.* **1979,** *16*, 466-475.

(5) Dwivedi, P.; Burns, R. B. The natural occurrence of ochratoxin A and its effects in poultry: A review. *World's Poult. Sci. J.* **1986,** *42*, 32-47.

(6) Creppy, E. E. Update of survey, regulation and toxic effects of mycotoxins in Europe. *Toxicol. Lett.* **2002,** 127, 19-28.

(7) Bennett, J. W.; Klich, M. Mycotoxins *Clinic. Microbiol. Rev.* **2003***, 16,* 497-516.

(8) Di Paolo, N.; Guarnieri, A.; Garosi, G.; Sacchi, G, Mangiarotti, A. M.; Di Paolo, M. Inhaled mycotoxins lead to acute renal failure. *Nephrol. Dial. Transplant* **1994,** *4,*116-120.

(9) Petrova-Bocharova, T.; Chernozemsky, I. N.; Castegnaro M. Ochratoxin A in human blood in relation to balkan endemic nephropathy and urinary system tumors in Bulgaria. *Food Addit. Contam.* **1988,** *5*, 299-231.

(10) Stoev, S. D. The role of ochratoxin A as a possible cause of Balkan endemic nephropathy and its risk evaluation . *Vet. Hum. Toxicol.* **1998,** *40*, 352-360.

(11) Chernozemsky, I. N.; Stoyanov, I. S.; Petkova-Bocharova, T. K.; Nicolov, I. G.; Draganov, I. V.; Stoichev, I. I.; Tanchev, Y.; Naidenov, D.; Kalcheva, N. D. Geographic correlation between the occurrence of edemic neophropathy and urinary tract tumors in vratza district, Bulgaria. *Int. J. Cancer.* **1977,** *19*, 1-11.

(12) European commission. Commission regulation No. 472/2002, 2002, Official J. European communities, L75, 18-20.

(13) European commission regulation 123/2005 of 26 January 2005 amending EC 466/2001 as regards ochratoxin A. L25, 3-5.

(14) Blesa, J.; Berrada, H.; Soriano, J. M.; Molto, J. C.; Manes, J. Rapid determination of ochratoxin A in cereals and cereal products by liquid chromatography. *J. Chromatogr. A* **2004,** *1046,* 127-131.

(15) Josefsson E.; Moller, T. High pressure liquid chromatography determination of ochratoxin A and zearalenone in cereals. *J. AOAC Int.* **1979,** *62*,1165-1168.

(16) Reinhard, H.; Zimmerli, B. Reversed-phase liquid chromatographyic behavior of mycotoxins citrinin and ochratoxin A. *J. Chromatogr. A* **1999,** *862*, 147-159.

(17) Romani, S.; Sacchetti, G..; Chaves L.; Pinnavaia, G, G.; Dalla R. M. Screening on the occurrence of ochratoxin A in green coffee beans of different origins and types, *J. Agric. Food Chem.* **2000,** *48*, 3616-3619

(18) Trucksess, M. W., Giler, J. Young, K. White, K. D., Page, S. W. Determination and survey of ochratoxin A in wheat, barley, and coffee-1997. *J. AOAC Int.* **1999,** *82*, 85-89.

(19) Barna-Vetro, I.; Solti, L.; Teren, J.; Gyongyosi, A.; Szabo, E.; Wolfling, A. Sensitive ELISA test for determination of ochratoxin A. *J. Agric. Food Chem.* **1996,** *44*, 4070-4074.

(20) Chu, F. S.; Chang, C. F; Hinsdill, R. D. Production of antibody against ochratoxin A. *Appl. Environ. Microbiol.* **1976,** *31* ,831-835.

(21) Clarke, J. R.; Marquardt, R. R.; Oosterveld, A.; Frohlich, A.; Madrid, F. J.; Dawood, M. Development of a quantitative and sensitive enzyme-linked immunosorbent assay for ochratoxin A using antibody from the Yolk of the laying hen. *J. Agric. Food Chem.* **1993,** *41*, 1784-1789.

(22) Kwak, B. Y.; Shon, D. H. Detection of ochratoxin A in agricultural commodities using enzyme-linked immunosorbent assay. *Food Sci. Biotechnol.* **2000,** *9,* 168-173.

(23) Pestka, J. J.; Steinert, R. W.; Chu, F. S. Enzyme-linked immunosorbent assay for detection of orchatoxin A in wheat. *Appl. Environ. Microbiol.* **1981,** *41,*1472-1474.

(24) Thirumala-Devi, K.; Mayo, M. A.; Reddy, G.; Reddy, S. V.; Delfosse, P.; Reddy D. V. R. Production of polyclonal antibodies against ochratoxin A and its detection in chilies by ELISA. *J. Agric. Food Chem.* **2000,** *48,* 5079-5082.

圖表

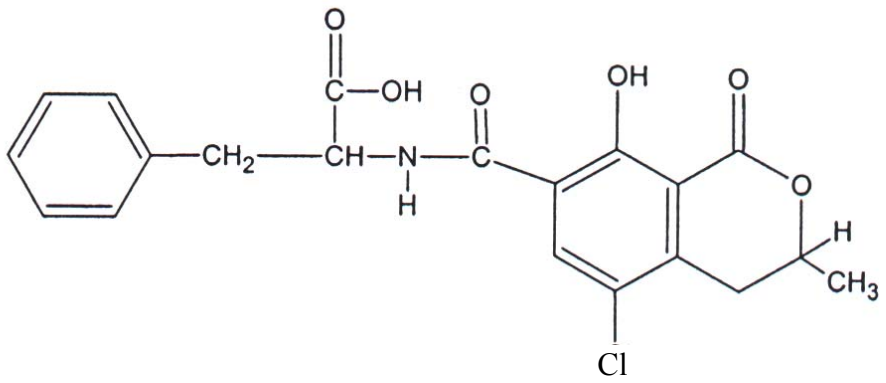


Figure 1. Structures of ochratoxin A

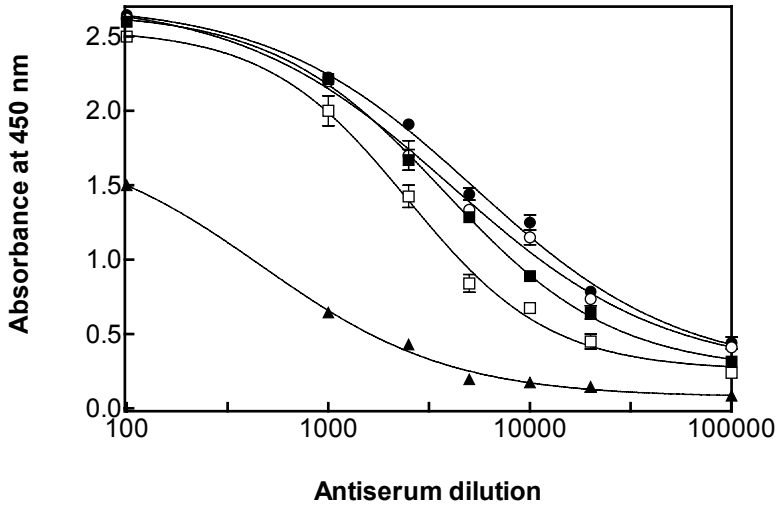
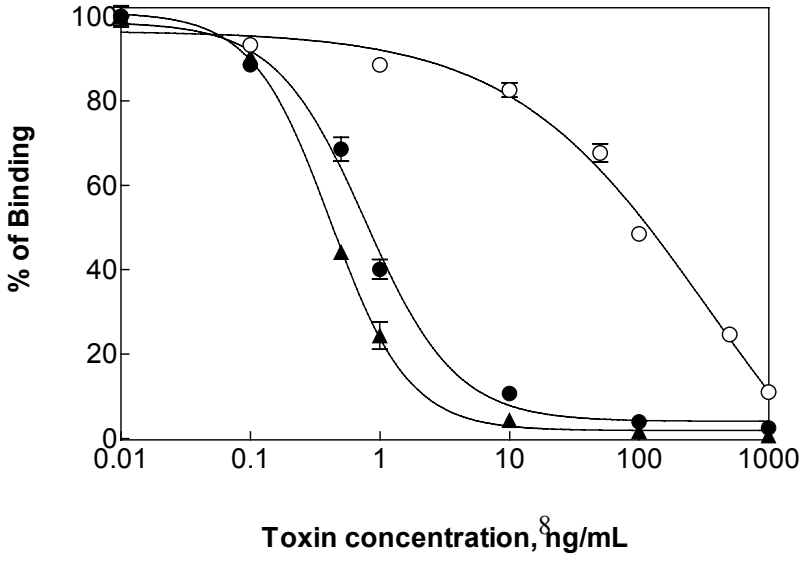


Figure 2. Determination of antibody titers for a representative rabbit after immunization with OTA-γ-globulin by an OTA-OVA based iELISA. The antiserum was obtained 0 (▲), 6 (□), 9 (■), 12 (○), and 15 (●) weeks after immunization.



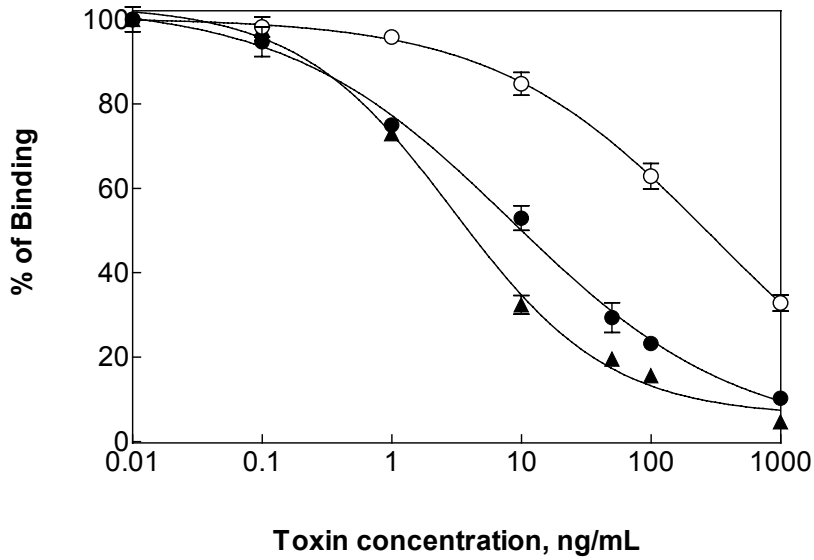


Figure 3. A. Cross-reactivity of anti-OTA antibodies with OTA (●), OTB (○) and OTC(▲) in a cdELISA. All data were obtained from the average of three sets of experiments. The absorbance of the control, A_0, with no toxin present, was 2.0; **B.** Cross-reactivity of anti-OTA antibodies with OTA (●), OTB (○) and OTC (▲) as determined by a ciELISA. All data were obtained from the average of three sets of experiments. The absorbance of the control, A_0, with no toxin present, was 1.8.

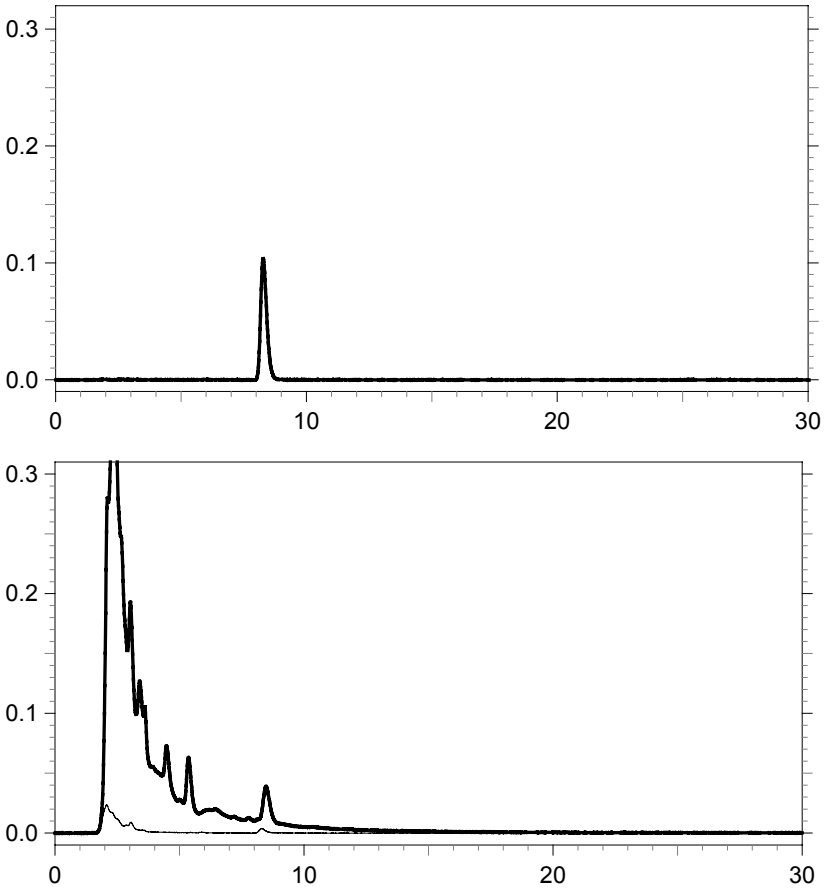


Figure 4. HPLC chromatograms of **A.** 5 ng of standard OTA and **B.** naturally contamination of OTA in coffee sample 20 (thick line) and buckwheat sample 10 (thin line).

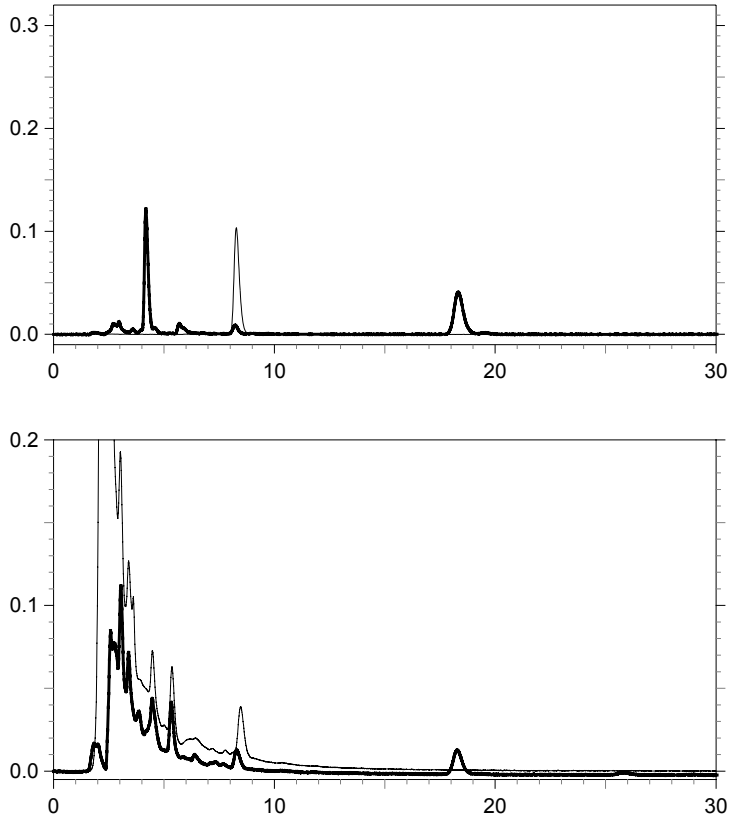


Figure 5. HPLC chromatograms of chemical derivatization of standard OTA and coffee sample. **A.** standard OTA (thin line) and methyl ester form of standard OTA (thick line). **B.** coffee sample containing OTA (thin line) and methyl ester form of OTA (thick line).

Table 1. ELISA and HPLC analysis of OTA in different agricultural commodities[a]

Samples	Source	ELISA OTA and analogs (ng/g ± SD)	HPLC OTA (ng/g)
corn			
1.	Taiwan	63 ± 5	ND
2.	Taiwan	24 ± 3	*
3.	Taiwan	ND	*
4.	Imported	16 ± 3	*
5.	Imported	27 ± 4	*
6.	Taiwan	ND	*
7.	Taiwan	30 ± 3	*
8.	Taiwan	ND	*
selected cereals			
9.	Imported wheat	84 ± 4	ND
10.	Imported buckwheat	144 ± 8	60
11	Imported barley	35 ± 3	*
12	Imported barley	43 ± 4	*
bean			
13	Taiwan (black bean)	74 ± 6	ND
14	Taiwan soybean	ND	*
15	Taiwan soybean	ND	*
16	Taiwan (red bean)	43 ± 5	ND
17	Taiwan (green bean)	ND	*
coffee			
18	Imported (green coffee)	ND	ND
19	Imported (green coffee)	ND	ND
20	Imported (roasted coffee)	160 ± 9	220

[a] Each sample was extracted twice and each extract was analyzed in triplicate

[b] ND, not detected or below the limit of detection

* Do not determine.