Studies on mycotoxin analysis using immunoaffinity column (2002 Japanese Association of Mycotoxicology Achievement Award)

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Summary

The traditional analysis of mycotoxins in food and feed required considerable time, large volume of solvents and often, special techniques. To overcome these problems, we developed the new analytical method for ochratoxin A (OTA) using the immunoaffinity column (IAC) specific for ochratoxin A (OTA) in 1990. The advantage of this method was one-step removal of interfering substances from samples. Moreover, the detection limit of 0.05 µg/kg for OTA in coffee beans was achieved. Using the IAC cleanup and HPLC with fluorometric or electrochemical detection, the surveys of aflatoxins (AFs) and OTA in green and roasted coffee beans, AFs in spices, and AFs, OTA, zearalenone (ZEN) and -zearalenol (-ZEL) in beers were conducted. With the use of IACs in the cleanup steps, the parts-per-trillion ranges of mycotoxins were detectable. The detection limits were 0.002 µg/kg for AFB₁ in coffee beans and spices, and 0.05 µg/kg for OTA in coffee beans, 0.0005 µg/L for AFB₁, 0.0010 µg/L for OTA, 0.020 µg/L for ZEN and -ZEL in beer samples. This paper also describes the effects of the elimination of fungi, AFB₁ and OTA in green coffee beans by handpicking.

Key words: immunoaffinity column, aflatoxins, ochratoxin A, -zearalenol, coffee beans, spice, beer

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Introduction

To minimize the interferences of co-extracted compounds from food and feed samples in which mycotoxins occur, the conventional chemical analyses for mycotoxins are highly time-consuming, need large amount of solvents, and mostly require practical experiences. From the environmental point of view, toxic solvents such as chlorinated solvents should be saved from now on. Under these circumstances, the immunoaffinity column (IAC) methods using specific antibodies against mycotoxins have been developed. The major advantages of the IAC are highly specific, simple, rapid, and saving toxic solvents. The first IAC for aflatoxins (AFs) was developed by Groopman *et al.*¹⁾ in 1984, and we first reported the IAC method for ochratoxin A (OTA)²⁾ in 1990. Recently, with the availability of commercial IACs for AFs, OTA, zearalenone (ZEN), fumonisins and deoxynivalenol (DON), these IACs have become the powerful tools in the cleanup stage of mycotoxin analysis. Moreover, because of the robustness, accuracy, and precision of IAC method, the AOAC International Official Methods of Analysis (OMA) has adapted 8 IAC methods for mycotoxin analysis.

Since the end of 1980s, we have surveyed mycotoxins in coffee beans, spices and beer samples

using the IAC cleanup. This paper summarizes those results, including the development of IAC specific for OTA and the effects of the elimination of fungi, AFB₁ and OTA in green coffee beans by handpicking.

Development of the IAC specific for OTA

For analyzing OTA in foods, most of the ordinary methods were time-consuming in the cleanup steps, and used a large volume of organic solvents such as chloroform. Moreover, it was difficult to detect low $\mu\text{g/kg}$ range of OTA in coffee samples by the conventional chemical methods, because coffee samples contain lots of fluorescent substances, which interfere TLC or HPLC-fluorometric (FL) detection. Under these circumstances, we developed the new analytical method for OTA using a monoclonal antibody (MAb)-IAC in the cleanup step^{2, 3)}. Main procedures of the IAC method were as follows. The anti-OTA MAb was coupled to Formyl-cellufine (Seikagaku Co., Tokyo, Japan), and then this immunosorbent was placed in a minicolumn. The capacity of IAC to bind OTA was 0.8-1.0 μg per 0.3 ml of the column. The coffee sample was extracted with 1 % sodium hydrogen carbonate solution, and filtered through a glass microfiber filter. The filtrate was applied onto the IAC. After washing the column with water, OTA was eluted with 30 % ethanol solution (pH 4.4). The eluate was subject to HPLC using an alkaline mobile phase. The advantage of this method was one-step removal of interfering substances from samples. Moreover, with the alkali-conditioned HPLC, the sensitivity was increased more than 100-fold compared to the conventional method. The detection limit of this method was 0.05 μg OTA/kg for coffee samples.

Survey of AFs and OTA in coffee beans

As for the contamination of AFs or OTA in green coffee beans, Levi *et al.*⁴⁾ first reported that 3 out of 68 samples were contaminated with 20-80 $\mu\text{g/kg}$ of OTA, and Levi⁵⁾ reported the contamination of AFB₁ in 2 out of 201 samples at 3 and 12 $\mu\text{g/kg}$. Tsubouchi *et al.*⁶⁾ also reported that 4 out of 22 samples were contaminated with 9.9-46 $\mu\text{g/kg}$ of OTA. A few papers described that caffeine in green coffee beans inhibited the growth of fungi and the production of mycotoxins^{7, 8)}. In contrast, however, Tsubouchi *et al.*^{6, 9)} reported that some strains of *Aspergillus ochraceus* and *A. flavus* isolated from green coffee beans grew well and produced high levels of OTA and AFB₁ in the presence of 0.1 %~1.0 % caffeine. Furthermore, it was demonstrated that OTA in green coffee beans was not degraded by roasting procedures, and the most OTA in beans transmitted into the beverage¹⁰⁾. In addition, Tsubouchi *et al.*¹¹⁾ first reported the presence of OTA in commercial roasted coffee beans in 5 out of 68 samples at a level of 3.2-17.0 $\mu\text{g/kg}$, at similar levels to green coffee beans reported previously. These results show that the contamination of mycotoxins in green coffee beans has the potential for affecting the human health.

The ordinary HPLC-FL detection of mycotoxins in coffee beans gives many interfering fluorescent peaks, which prevents detection of minor amounts of mycotoxins. However, with the introducing of the IAC in the cleanup steps, the HPLC profiles showed no significant interfering peaks (Fig.1), and allowed detection of the parts-per-trillion levels of AFB₁ and OTA in green coffee beans.

Fig. 1. Chromatograms of (a) AFB₁ in green coffee beans, 0.033 ppb and (b) OTA in green coffee beans, 2.1 ppb.

Using the IAC cleanup followed by HPLC-FL detection, commercial green and roasted coffee beans were surveyed for AFs and OTA. As shown in Table 1, AFB₁ was detected in 27 out of 96 green coffee samples at a level of 0.002-0.033 $\mu\text{g/kg}$, and not detected from roasted coffee samples. OTA was detected in 50 out of 121 green coffee samples at a level of 0.07-72.68 $\mu\text{g/kg}$, and also detected in 4 out of 26 roasted coffee samples at a level of 0.10-8.91 $\mu\text{g/kg}$ (Table 2). The samples contaminated with OTA and/or AFB₁ were mainly products imported from African and Asian countries. The mycological survey revealed that members of the *A. niger* group were prominent in the mycoflora of the green coffee beans examined (data not shown). So, OTA production by the *A. niger* group isolated from green coffee beans was examined. The data revealed that 2 out of 30 isolates from samples produced 0.56 and 0.08 $\mu\text{g/kg}$ of OTA in rice culture, so it is possible to assume that the *A. niger* group is partly responsible for the OTA contamination in coffee beans.

Table 1. Aflatoxins (AFs) in commercial green and roasted coffee beans

Sample	No. of samples	No. of positives	Range ($\mu\text{g/kg}$)		
			AFB ₁	AFB ₂	AFG ₁
Green coffee beans	96	27	0.002-0.033	0.004-0.006	0.012
Roasted coffee beans	18	0	ND ^a	ND	ND

^a ND = not detected (less than detection limit of 0.002 $\mu\text{g AFs/kg}$).

Table 2. Ochratoxin A (OTA) in commercial green and roasted coffee beans

Sample	No. of samples	No. of positives	Range of OTA ^a ($\mu\text{g/kg}$)
Green coffee beans	121	50	0.07-72.68
Roasted coffee beans	26	4	0.10-8.91

^a Detection limit is 0.05 $\mu\text{g/kg}$

Elimination of mycotoxins in green coffee beans by handpicking

Because of the heat stability of AFs and OTA^{10, 12)}, it is very important how to eliminate the mycotoxins in green coffee beans. We examined the effect of the elimination of fungi, and AFB₁ and OTA in green coffee beans by the handpicking method to remove the defective beans that make bad taste of coffee beverage¹³⁾. Each green coffee beans sample imported from Indonesia, Yemen, Tanzania, and Brazil was divided into 2 groups; 1) the good beans after handpicking, 2) the bad beans, which were the defective beans such as worm-eaten spot beans, mold beans, shell beans, dead beans, discoloration beans, fermentation beans, parchment beans after handpicking. AFB₁ and OTA were analyzed by the IAC cleanup and HPLC-FL detection. As showed in Table 3, the mean numbers of fungi in the good beans were about two-third to one-tenth of that of the bad beans. This handpicking method also reduced the mean levels of both AFB₁ and OTA in the good beans compared to bad beans (Table 4, 5). In this way, the handpicking method removing the defective beans was very effective to eliminate the mycotoxins and fungi in green coffee beans.

Table 3. Numbers of fungi in green coffee beans after handpicking

Country	No. of samples	Mean No. of fungi (range × 10 ² CFU ^a /g)	
		Good beans	Bad beans
Indonesia	6	400 (0.3-840)	600 (0.8-1250)
Yemen	6	100 (0.8-530)	940 (1.3-5500)
Tanzania	5	20 (1-56)	66 (18-220)
Brazil	5	1.4 (0-2.5)	13 (3.2-30)

^a CFU = colony forming units

Table 4. Contamination levels of aflatoxin B₁ (AFB₁) in green coffee beans after handpicking

Country	Good beans		Bad beans	
	Incidence	Mean levels of AFB ₁ ^a (range, µg/kg)	Incidence	Mean levels of AFB ₁ (range, µg/kg)
Indonesia	3/6	0.002 (0.004-0.006)	4/6	0.022 (0.003-0.101)
Yemen	2/6	0.001 (0.002, 0.004)	3/6	0.004 (0.002-0.018)
Tanzania	0/5	– ^b	1/5	0.0004 (0.002)
Brazil	0/5	–	0/5	–

^a Mean values were calculated by assuming that samples containing levels less than detection limit (0.002 µg/kg) contained AFB₁ at 0 µg/kg.

^b – = not applicable.

Table 5. Contamination levels of ochratoxin A (OTA) in green coffee beans after handpicking

Country	Good beans		Bad beans	
	Incidence	Mean levels of OTA ^a (range, μ g/kg)	Incidence	Mean levels of OTA (range, μ g/kg)
Indonesia	2/6	0.04 (0.08, 0.13)	4/6	0.86 (0.38-4.78)
Yemen	4/6	1.75 (0.22-7.67)	6/6	21.04 (2.73-72.70)
Tanzania	1/5	0.03 (0.13)	2/5	0.74 (0.69, 3.00)
Brazil	0/5	– ^b –	1/5	0.02 (0.08)

^a Mean values were calculated by assuming that samples containing levels less than detection limit (0.05 μ g/kg) contained OTA at 0 μ g/kg.

^b – = not applicable.

Survey of fungi and AFs in spices

Recently, with a change of the Japanese favorite foods, their consumption of spices has increased. In Japan, more than 98 % of spices are imported, and their origins are tropical, sub-tropical countries where AFs contamination may be expected to occur. In Japan, only a few papers have described the contamination of AFs with a limited survey of each spice. In general, the contamination levels of AFs in spices are low except for chilli, pepper and nutmeg. However, many aflatoxigenic *A. flavus* are isolated from the spices, the production of AFs may occur when the fungi survive and multiply under favorable conditions. Under these circumstances, we surveyed the fungi and AFs in imported spices. Two hundred and fifty-seven samples were collected in Nagoya City from 1990 to 2001, and these samples were divided into seven types such as fruits, seeds, leaves, roots, barks, flowers, and mixed spices (Table 6). The most spices contain lots of fluorescent substances that interfere AFs analysis by HPLC-FL detection. By introducing the IAC method into the cleanup stage, no interfering peaks appeared on the chromatogram of HPLC, and the detection limit of AFB₁ was achieved at 0.002 μ g/kg.

Though about 50 % of spices had total fungal counts of less than 10² CFU/g, about 17 % of spices, which were mainly fruits, leaves and flowers, had total fungal counts of more than 10⁴/g. Six AF-producing strains of *A. flavus* were isolated from the spices, such as cayenne pepper, paprika, black pepper, and gharum masala. These strains produced AFB₁ and B₂, moreover, one strain from paprika produced AFB₁, B₂, G₁, and G₂ (data not shown). As shown in Table 6, AFB₁ was detected in 47 out of 257 samples (18.3 %) with the range of 0.002-8.7 μ g/kg. AFB₁, B₂ and G₁ was detected from fruits spices such as white pepper, paprika, and nutmeg, and AFB₁, B₂, G₁, and G₂ were detected from one sample of paprika. A high incidence of AFB₁ was occurred in fruits spices (25.7 %) such as red pepper, black pepper, paprika and nutmeg, and in mixed spices (88.8 %). A low incidence of AFB₁ was occurred in seeds, leaves and barks spices, and these contamination levels were equal or less than 0.09 μ g/kg.

Table 6. Incidence and levels of aflatoxins (AFs) in Spices

	Spice	No. of samples	No. of positives	Range ($\mu\text{g/kg}$)			
				AFB ₁	AFB ₂	AFG ₁	AFG ₂
Fruits	Red pepper	16	5	0.007-0.595	0.007-0.031	ND ^a	ND
	Pink pepper	1	1	0.007	ND	ND	ND
	Black pepper	34	6	0.003-0.726	0.052-0.350	ND	ND
	White pepper	14	5	0.002-0.265	0.003-0.012	0.007	ND
	Paprika	5	3	0.043-3.230	0.137-0.005	0.996	0.040
	Nutmeg	17	5	0.055-7.770	0.013-0.780	0.038	ND
	Mace	10	1	0.010	ND	ND	ND
	Cayenne pepper	1	1	0.007	ND	ND	ND
	Chinese pepper	6	0	ND	ND	ND	ND
	Juniper berry	1	0	ND	ND	ND	ND
Total		105	27				
Seeds	Coriander	12	3	0.012-0.090	0.007-0.008	ND	ND
	Cardamon	11	1	0.002	ND	ND	ND
	Cumin	11	1	0.017	ND	ND	ND
	Fennel	11	0	ND	ND	ND	ND
	Poppy seed	3	1	0.084	0.008	ND	ND
	Others	4	0	ND	ND	ND	ND
Total		52	6				
Leaves	Thyme	11	1	0.007	ND	ND	ND
	Sage	10	0	ND	ND	ND	ND
	Oregano	11	0	ND	ND	ND	ND
	Marjoram	11	0	ND	ND	ND	ND
	Rosemary	11	0	ND	ND	ND	ND
	Others	5	0	ND	ND	ND	ND
Total		59	1				
Roots	Turmeric	2	2	0.027-0.100	ND	ND	ND
	Ginger	15	2	0.003	ND	ND	ND
	Others	4	0	ND	ND	ND	ND
Total		21	5				
Barks	Cinnamon	10	0	ND	ND	ND	ND
Mixed spices	Chilli powder	3	3	1.470-8.700	0.134-0.170	ND	ND
	Curry powder	5	4	0.050-0.302	0.010-0.045	ND	ND
	Gharum masala	1	1	0.079	0.01	ND	ND
Total		9	8				
Flowers	Cloves	1	0	ND	ND	ND	ND
Total		257	47				

^a ND = not detectable (less than detection limit of 0.002 AFs $\mu\text{g/kg}$).

Survey of mycotoxins in imported and domestic beer

The possibility of mycotoxins getting into beer from contaminated grains used in brewing was pointed out in the early 1970s, and mycotoxins such as AFs, OTA, ZEN and DON in beer recently reviewed¹⁴⁾. Since 1994, with the use of IAC in the cleanup steps, detection limit has improved to the

parts-per-trillion range, and many incidences of OTA contamination of beer have been reported, although levels were very low. Also the parts-per-trillion levels of AFs have been found in Mexican and Brazilian beers¹⁵⁾. On the other hand, ZEN has not been detected in beer samples in recent surveys. As that reason, some papers have reported that ZEN has been converted to its related compounds, such as *-zearalenol* (*-ZEL*), *-zearalenol* (*-ZEL*), and zearalanone (ZAN), by brewing strains *Saccharomyces cerevisiae*. So, it is necessary to analyze not only ZEN but also *-ZEL* in beer samples.

In 1998, we surveyed OTA and AFs in 94 imported beer samples from 31 producing countries and in 22 Japanese beer samples by using the IAC cleanup and HPLC-FL detection¹⁶⁾. The detection limits were 0.0010 $\mu\text{g/L}$ for OTA, 0.0005 $\mu\text{g/L}$ for AFs B₁ and B₂, and 0.0010 $\mu\text{g/L}$ for AFs G₁ and G₂. OTA was detected in 86 out of 94 imported beer samples at a mean level of 0.0101 $\mu\text{g/L}$ and in 21 out of 22 Japanese beer samples at a mean level of 0.0125 $\mu\text{g/L}$. AFB₁ was detected in 11 out of 94 imported beer samples at a level of 0.0005-0.0831 $\mu\text{g/L}$ and 2 out of 22 Japanese beer samples at 0.0005 and 0.0008 $\mu\text{g/L}$ (data not shown). Although OTA was detected in most samples from various countries, AFs were detected in the beer samples from only a limited number of countries where AF contamination might be expected to occur because of their warm climate.

In 2000, we surveyed AFs, OTA, ZEN, and *-ZEL* in beer samples. In the ordinal analysis of ZEN, HPLC-fluorescence detection has been widely used, however, the fluorescence intensity of *-ZEL* is very low, and other related compounds such as *-ZEL* or ZAN cannot be detected. However, the introduction of the IAC cleanup and electrochemical detection to HPLC increased the sensitivity of ZEN and its related compounds. The detection limit of ZEN and its related compound was achieved at 0.020 $\mu\text{g/L}$.

As shown in Table 7, ZEN was detected in 3 samples only from American beer at a level of 0.034-0.069 $\mu\text{g/ml}$. *-ZEL* was detected in 29 out of 94 imported beer samples at a level of 0.020-0.838 $\mu\text{g/ml}$ and 2 out of 14 Japanese beer samples at 0.099 and 0.174 $\mu\text{g/ml}$. High incidence of *-ZEL* contamination was observed in North, Central, and South American beer samples. The contamination levels and incidences of OTA and AFs were the same as the former study as follows. OTA was detected in 86 out of 94 imported beer samples at a mean level of 0.0178 $\mu\text{g/L}$ and in 12 out of 14 Japanese beer samples at a mean level of 0.0161 $\mu\text{g/L}$. AFB₁ was detected in 16 out of 94 imported beer samples at a level of 0.0009-0.0266 $\mu\text{g/L}$ and 1 out of 22 Japanese beer samples at 0.0007 $\mu\text{g/L}$ (data not shown).

Concluding remarks

The above analytical results demonstrate that the IAC method is well suited for the detection of mycotoxins at the parts-per-trillion level. With the availability of commercial IACs for mycotoxins, IAC method has emerged as an important cleanup technique in the mycotoxin analysis. Moreover, as the OMA of AOAC International has already adapted 8 IAC methods for mycotoxin analysis, the IAC method gives better precision and accuracy (e.g., RSD_R or HORRAT value) than that of conventional methods. The IAC method is expected to occupy an important position in mycotoxin analysis from now on increasingly.

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Table 7. Zearalenone (ZEN), α -zearalenol (α -ZEL) in imported and Japanese beer samples

Region or country	ZEN		α -ZEL	
	Incidence	Range ($\mu\text{g/L}$)	Incidence	Range ($\mu\text{g/L}$)
North America	3/19	0.034-0.069	10/19	0.029-0.838
Canada	0/3	ND ^a	0/3	ND
United States	3/16	0.034-0.069	10/16	0.029-0.838
Central America	0/8	ND	4/8	0.182-0.357
Jamaica	0/1	ND	0/1	ND
Mexico	0/6	ND	4/6	0.182-0.357
Trinidad	0/1	ND	0/1	ND
South America	0/3	ND	2/3	0.026, 0.042
Brazil	0/2	ND	1/2	0.026
Peru	0/1	ND	1/1	0.042
Oceania	0/8	ND	0/8	ND
Australia	0/3	ND	0/3	ND
New Zealand	0/4	ND	0/4	ND
Tahiti	0/1	ND	0/1	ND
Africa	0/2	ND	1/2	0.022
Kenya	0/1	ND	1/1	0.022
South Africa	0/1	ND	0/1	ND
Europe	0/40	ND	10/40	0.020-0.160
Austria	0/1	ND	0/1	ND
Belgium	0/7	ND	1/7	0.160
Denmark	0/3	ND	1/3	0.061
France	0/3	ND	0/3	ND
Germany	0/11	ND	2/11	0.022, 0.027
Holland	0/3	ND	0/3	ND
Italy	0/3	ND	3/3	0.020-0.064
Portugal	0/2	ND	2/2	0.027, 0.036
United Kingdom	0/7	ND	1/7	0.081
Asia	0/14	ND	3/14	0.023-0.093
China	0/1	ND	0/1	ND
Indonesia	0/2	ND	0/2	ND
Israel	0/1	ND	1/1	0.093
Philippines	0/2	ND	1/2	0.023
Singapore	0/2	ND	0/2	ND
Taiwan	0/1	ND	1/1	0.041
Thailand	0/3	ND	0/3	ND
Vietnam	0/2	ND	0/2	ND
Over all	3/94	0.034-0.069	29/94	0.020-0.838
Japan	0/14	ND	2/14	0.099, 0.174

^a ND = not detectable (less than detection limit of 0.020 ZEN and α -ZEL $\mu\text{g/L}$).

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マイコトキシン分析へのイムノアフィニティーカラムの応用に関する研究（学術奨励賞）

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著者らは以前よりコーヒー豆及びコーヒー製品のオクラトキシン A（OTA）汚染調査を行っていたが、コーヒーに含まれている多量の夾雑物質のため、微量な OTA を検出するのに煩雑な前処理操作が必要であった。そこで著者らは OTA に特異的なイムノアフィニティーカラム(IAC)を作製し、コーヒー中の OTA 分析の前処理に応用したところ、その微量定性分析における有用性が判明した。この IAC により試料中の OTA はワンステップで精製、濃縮することができ、HPLC- 蛍光検出において $0.05 \mu\text{g}/\text{kg}$ の OTA が検出可能となった。著者らはこれまでに、自作の OTA に特異的な IAC および市販の IAC を用いて、コーヒー豆のアフラトキシン (AFs) および OTA 汚染調査、香辛料の AFs 汚染調査、ビールの AF, OTA, ゼアラレノン (ZEN) および -ゼアラレノール (-ZEL) 汚染調査を行ってきた。これらの調査において、試料からのマイコトキシンの分離・精製に IAC を応用することにより、分析操作は簡便になり、しかも極微量 (ppt レベル) のマイコトキシンを検出することが可能になった。そこで今回は上記の研究結果に加え、選別によるコーヒー豆のカビおよびマイコトキシン除去について述べる。

キーワード：イムノアフィニティーカラム，アフラトキシン，オクラトキシン A，-ゼアラレノール，
コーヒー豆，香辛料，ビール