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Talanta 69 (2006) 650–655
Talanta
www.elsevier.com/locate/talanta

# The comparison of two clean-up procedures, multifunctional column and immunoaffinity column, for HPLC determination of ochratoxin A in cereals, raisins and green coffee beans

Yoshiko Sugita-Konishi [a,*], Toshitugu Tanaka [b], Masahiro Nakajima [c], Kazuhiro Fujita [d], Hiroko Norizuki [e], Naoki Mochizuki [f], Kosuke Takatori [a]

[a] *Division of Microbiology, National Institute of Health Sciences, 1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan*
[b] *Kobe Institute of Health, 4-6, Minatojima-nakamachi, Chuo-ku, Kobe 650-0046, Japan*
[c] *Nagoya City Public Health Research Institute, 1-11, Hagiyama, Mizuho-ku, Nagoya 467-8615, Japan*
[d] *Japan Food Research Laboratories, Nagoya Branch, 4-5-13, Otu, Naka-ku, Nagoya 460-0011, Japan*
[e] *Japan Grain Inspection Association, 2-17-3, Arai, Ichikawa-shi, Chiba 272-0144, Japan*
[f] *Asahi Breweries Ltd., 1-1-21, Midori, Moriya-shi, Ibaraki 302-0106, Japan*

Received 15 September 2005; received in revised form 26 October 2005; accepted 26 October 2005
Available online 7 December 2005

**Abstract**

To evaluate a clean-up method of detecting ochratoxin A (OTA) by HPLC, the performances of two different clean-up columns, an immunoaffinity column and a multifuntional column were compared in an inter-laboratory study. As samples, un-contaminated wheat, corn grits, green coffee beans and naturally contaminated raisins were used. The recovery test was performed at two different concentrations of OTA (0.5 and 5.0 μg/kg) except for naturally contaminated raisins. Using the immunoaffinity column, the recovery rates, and relative standard deviations for repeatability ($R.S.D._r$) and reproducibility ($R.S.D._R$) for wheat, corn grits and green coffee beans ranged 59.0–85.8, 4.2–7.8 and 22.9–29.2%, respectively. For naturally contaminated raisins, recovery, $R.S.D._r$ and $R.S.D._R$ were 84.1, 1.8 and 5.1%, respectively. Using the multifunctional column, the recovery rates, $R.S.D._r$ and $R.S.D._R$ for wheat, corn grits and green coffee beans ranged 80.8–185.0, 0.7–6.9 and 15.2–33.9%, respectively. For naturally contaminated raisins, the recovery, $R.S.D._r$ and $R.S.D._R$ were 128.7, 1.1 and 3.7%, respectively. The results suggest that a multifunctional column could be used to detect OTA in wheat and corn grits at a concentration as low as 0.5 μg/kg; however, it was difficult to detect OTA in green coffee beans and raisins at such a low level. Although an immunoaffinity column could be used for all the test samples in this study from a low level to a high level, the recovery rates were lower than with a multifunctional column.


*Keywords:* Ochratoxin A; Immunoaffinity column; Multifunctional column; HPLC; Clean-up

## 1. Introduction

Ochratoxin A (OTA) produced by *Aspergillus ochraceus*, *Aspergillus carbonarius* and *Penicillium verrucosum* is a chlorinated isocoumarin compound. Among mycotoxins contaminated in food, drink and feed, OTA is one of the most widespread and hazardous mycotoxins. Risk assessments carried out by Joint FAO/WHO Expert Committee on Food Additives and Commission of the European Union [1,2] have shown that OTA has nephrotoxicity, teratogenic toxicity, immunotoxicity, genotoxicity and carcinogenicity. OTA has also been identified in human breast milk and blood, and has a long half-life in mammalian tissues [3–5]. Ueno et al. [5] reported detecting OTA in blood of Japanese. Therefore, exposure to OTA is common and serious problem in food safety.

Dietary sources of OTA has been found, in many commodities, such as coffee [6,7], beer [8,9], dried fruit [10,11], wine [12,13], cereals [14,15], cocoa [16,17], spices [18,19] and nuts [20].

Many countries have established maximal limits for OTA in food for enforcement. It is essential to develop and validate

* Corresponding author. Tel.: +81 3 3700 1141; fax: +81 3 3700 9527.
*E-mail address:* ykonishi@nihs.go.jp (Y. Sugita-Konishi).

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doi:10.1016/j.talanta.2005.10.036

analytical methods for the detection of OTA in individual foodstuff reliably, speedy and simply.

There are numerous methods for the determination of OTA in food-stuffs, such as thin layer chromatography, enzyme-linked immunosorbent assay, high performance liquid chromatography and liquid chromatography–mass spectroscopy. As regards clean-up methods, the demand for immunoaffinity column and multifunctional column has increased for mycotoxin analysis in recent years [21]. Although there are many collaborative studies using immunoaffinity column [22–25], there is little information about multifunctional clean-up column for OTA in individual material [26].

In this paper, the performances of two different clean-up procedures, using an immunoaffinity and a multifunctional column, respectively, were compared in an inter-laboratory study involving six laboratories. Food materials used in this study were wheat, corn grits, raisins and green coffee beans. Except naturally contaminated raisins, all materials were spiked with a low (0.5 μg/kg) or high (5.0 μg/kg) concentration of OTA. The analytical methods were assessed based on parameters of recovery and the precision.

## 2. Experimental

### 2.1. Standard and reagents

The stock standard solution was prepared from crystalline OTA (Wako Pure chemical Industries Ltd., Osaka, Japan) at a concentration of ca. 40 μg/mL. The concentration was checked by the method recommended by AOAC International [27], then a working standard solution (2 μg/mL) was prepared in toluene–acetic acid (99 + 1).

The spiking solutions of OTA (1.25 and 0.125 μg/mL) for the recovery test were prepared by dilution with acetonitrile. Water, acetonitrile and methanol of HPLC grade and the other analytical grade reagents were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Multisep #229 was purchased from Romer Labs Inc. (Stylemaster Drive Union, MO, USA). The immunoaffinity column (OchraTest WB) was purchased from VICAM (Watertown, MA, USA). Phosphate-buffered saline (PBS) tablets and Tween 20 were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

### 2.2. Preparation of samples

Naturally contaminated raisins which was homogenized type, used in the Food Analytical Performance Assessment Scheme (FAPAS) Program carried out in 2003, were purchased from Central Science laboratories (York, UK). The assigned concentration of the sample was 13.2 μg/kg according to the FAPAS report. Blank green coffee beans, wheat and corn grits were obtained from commercial sources and previously shown to contain <0.2 μg/kg of OTA using the AOAC official method [25] and the method of Nakajima et al. [28]. All samples except raisins were ground and passed though a mesh of 1 mm. After mixing and homogenizing, they were packed into a bag at 30 g each.

### 2.3. Fortification procedure

For evaluating recovery, 100 μL of each spiking solution was added to 25 g of "blank" material in a 200-mL flask (final concentration, 0.5 and 5 μg/kg) and kept at room temperature. After 1 h, the spiked sample was extracted using each clean-up method.

### 2.4. Clean-up with the immunoaffinity column

Twenty-five grams of sample was weighed in a 200-mL glass-stopped Erlenmeyer flask, and extracted with 100 mL of a methanol/aqueous 1% $NaHCO_3$ solution (70:30, v/v). The suspension was shook vigorously for 30 min on a wrist action shaker and passed through a Whatman No. 4 paper filter. Then 8 mL of the filtrate was diluted to 100 mL in PBS containing 0.01% Tween 20 (PBS-Tween) and passed through a Whatman 934 AH glass filter. Fifty milliliters of the diluted extract, equivalent to 1.0 g of sample, was applied to an OchraTest column under gravity. The column was washed with 3 mL of PBS-Tween followed by water, and OTA was eluted with 3 mL of methanol into a silanized vial, at a flow rate of one drop/s. Then the eluate was evaporated to dryness under nitrogen gas at 40 °C.

### 2.5. Clean-up with multifunctional column

Twenty-five grams of sample was weighed accurately in a 200-mL flask and extracted with 100 mL of acetonitrile–water (84 + 16) by shaking for 30 min on a wrist action shaker. The extract was passed through a Whatman 934AH glass filter, 10 mL of the filtrate was transferred into a new test tube, and 100 μL of acetic acid (analytical grade, 99.7%) was added. After mixing well, the acetic extract was applied to a #229 column. The first 5 mL of eluate was collected and 4.0 mL, equivalent to 1.0 g of sample, was evaporated to dryness under nitrogen gas at 40 °C.

### 2.6. Conditions for HPLC

The residues obtained with both clean-up procedures were dissolved in 1.0 mL of acetonitrile:water:acetic acid (30:70:1), and the purified sample solution was subjected to an HPLC analysis. When the final sample solution was cloudy, the solution was filtered through a 0.45 um pore size PTFE syringe filter before analysis by HPLC. The conditions for HPLC are listed in Table 1. The analytical ODS column (100–250 mm × 4.6 mm i.d.) was kept at 45 °C and a mobile phase of acetonitorile–water–acetic acid (55:43:2 v/v) was delivered at a rate of 1.0 mL/min.

Table 1
HPLC conditions for detection of OTA

| |
|---|
| Column: ODS 250 mm × 4.6 mm, i.d. (3–5 μm) |
| Column temperature: 45 °C |
| Flow rate: 1.0 mL/min |
| Wavelength: Ex 333 nm, Em 460 nm |
| Injected volumn: 100 μL |
| Mobile phase: $CH_3CN$–$H_2O$–$CH_3COOH$ (55:43:2, v/v) |

Detection was performed with a fluorescence detector (excitation wavelength 333 nm and emission wavelength 460 nm). A seven-point calibration curve (0.2–20.0 ng/mL) covering the range of interest for the test sample was established. The calibration curve was to be linear.

### 2.7. Inter-laboratory study

To evaluate and compare the two clean-up procedures, an inter-laboratory study was carried out using 11 materials (naturally contaminated raisins, two spiked samples of unpolished wheat, two spiked samples of green coffee beans, two spiked samples of corn grits and a blank of each material) in six laboratories within Japan.

### 2.8. Statistics

Statistical evaluation was performed using the results from the six laboratories (except the results for green coffee beans obtained using the multifunctional column) and parameters of precision, that is, the inter-laboratory relative standard deviation for repeatability ($R.S.D._r$) and for reproducibility ($R.S.D._R$), were deduced as recommended by the AOAC [29].

## 3. Results and discussion

### 3.1. Analytical results using the immunoaffinity clean-up column

When the sample was cleaned-up with the immunoaffinity procedure, a clear base line for the chromatogram was obtained from the blanks of all samples used in this study (Fig. 1(a, d and g)).

In all samples spiked with 0.5 and 5 μg/kg of OTA, the peak of OTA was sharp and isolated from other peaks. The limit of determination (LOD) for all materials used in this study (wheat, corn, green coffee beans and raisins) was estimated at 0.1 μg/kg, which was calculated as an *S/N* ratio of 3/1.

As shown in Table 2, the mean recovery of OTA from wheat spiked at a level of 0.5 and 5 μg/kg was 79.6 and 81.5%, from corn spiked at a level of 0.5 and 5 μg/kg was 79.0 and 85.8%, and from green coffee beans spiked at a level of 0.5 and 5 μg/kg was 64.6 and 59.0%, respectively. In naturally contaminated raisins, the mean concentration of OTA was 11.1 μg/kg, and the recovery was 84.1%, which was calculated based on the mean average reported by FAPAS. Except for green coffee beans, the recovery was over 78%. In cereals, the relative standard deviation ($R.S.D._r$) obtained within the six laboratories ranged from 4.2 to 6.8% and the relative standard deviation obtained between laboratories ($R.S.D._R$) ranged from 22.9 to 24.9%. In green coffee beans, the $R.S.D._r$ and $R.S.D._R$ were 5.9, 7.8, 24.6 and 29.2%, respectively. In naturally contaminated raisins, the $R.S.D._r$ and $R.S.D._R$ were 1.8 and 5.1%, respectively. Regarding to IUPAC/AOAC/ISO/CEN standards, for an analytical method of detecting mycotoxins to be recognized as an official standard for the purpose of enforcement, the recovery and the $R.S.D._r$ and $R.S.D._R$ values are required to be in the range of 70–110, <20 and <30%, respectively [30]. The results with the immunoaffinity clean-up procedure satisfied these criteria except for green coffee beans. In green coffee beans, although the recovery was below to 70% at both concentrations (0.5 and 5.0 μg/kg), the $R.S.D._r$ and $R.S.D._R$ values were less than 20 and 30%,

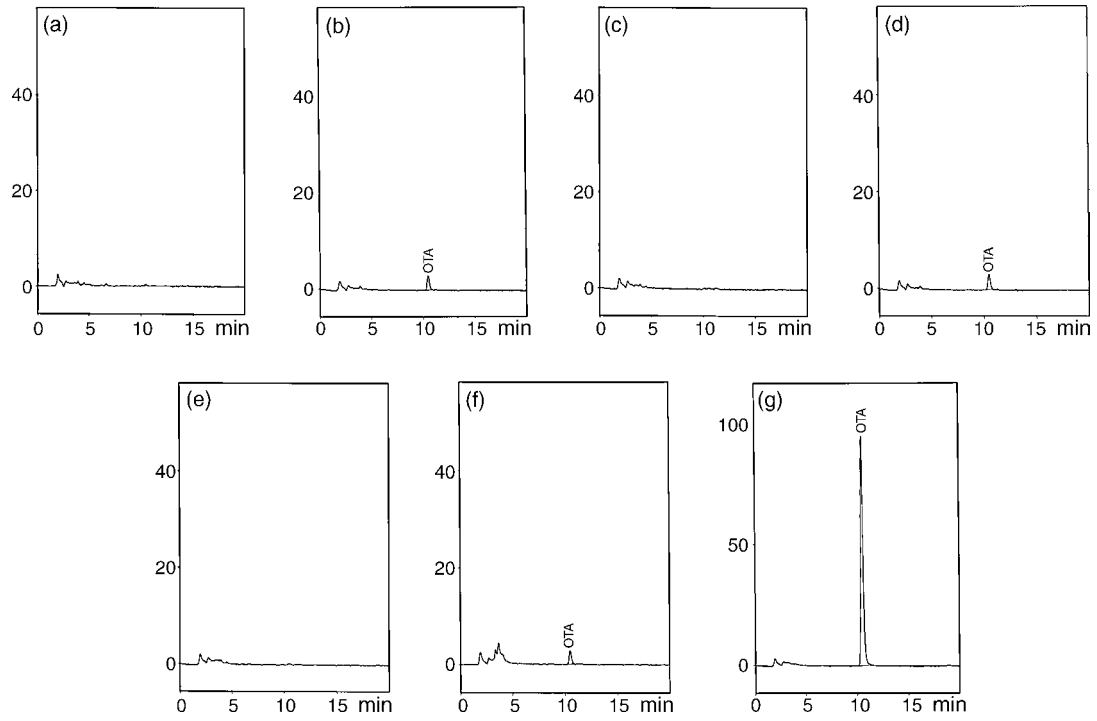


Fig. 1. Chromatograms of OTA detected in food samples using an immunoaffinity clean-up column: (a) blank wheat, (b) wheat spiked with 0.5 μg/kg of OTA, (c) blank corn, (d) corn spiked with 0.5 μg/kg of OTA, (e) blank green coffee beans, (f) green coffee beans spiked with 0.5 μg/kg of OTA, (g) naturally contaminated raisins. For chromatographic conditions see text.

Table 2
Results of an interlaboratory study using an immunoaffinity clean-up column for detection of OTA

| Number of laboratory | Spiked wheat | | | | Spiked corn | | | | Spiked green coffee beans | | | | Naturally contaminated raisins | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0.5 μg/kg | | 5.0 μg/kg | | 0.5 μg/kg | | 5.0 μg/kg | | 0.5 μg/kg | | 5.0 μg/kg | | | |
| 1 | 78.0 | 82.0 | 88.2 | 88.8 | 86.0 | 80.0 | 95.2 | 83.2 | 78.0 | 84.0 | 68.8 | 72.0 | 13.76 | 13.73 |
| 2 | 72.0 | 70.0 | 78.8 | 73.0 | 88.0 | 78.0 | 81.6 | 84.0 | 58.0 | 64.0 | 65.2 | 56.0 | 9.78 | 9.88 |
| 3 | 77.0 | 74.0 | 76.3 | 68.0 | 71.2 | 70.2 | 73.8 | 75.2 | 51.2 | 60.4 | 49.2 | 50.6 | 8.11 | 8.59 |
| 4 | 86.0 | 78.0 | 85.8 | 90.6 | 60.0 | 60.0 | 88.0 | 72.0 | 40.0 | 46.0 | 41.6 | 47.8 | 11.88 | 12.11 |
| 5 | 80.0 | 72.0 | 71.6 | 70.2 | 86.0 | 80.0 | 87.4 | 88.6 | 58.0 | 68.0 | 51.4 | 54.0 | 9.50 | 9.21 |
| 6 | 92.0 | 94.0 | 95.0 | 91.6 | 96.0 | 92.0 | 99.2 | 101.4 | 84.0 | 84.0 | 75.0 | 76.0 | 13.55 | 13.71 |
| Mean value (μg/kg) | | | | | | | | | | | | | 11.1 | |
| Mean recovery (%) | 79.6 | | 81.5 | | 79.0 | | 85.8 | | 64.6 | | 59.0 | | 84.1 | |
| Precision (%) | | | | | | | | | | | | | | |
| $S_r$ | 3.7 | | 3.4 | | 4.0 | | 5.9 | | 4.9 | | 3.5 | | 0.2 | |
| R.S.D.$_r$ | 4.8 | | 4.2 | | 5.0 | | 6.8 | | 7.8 | | 5.9 | | 1.8 | |
| $S_R$ | 18.2 | | 19.8 | | 19.7 | | 21.3 | | 18.9 | | 14.5 | | 0.6 | |
| R.S.D.$_R$ | 22.9 | | 24.4 | | 24.9 | | 24.8 | | 29.2 | | 24.6 | | 5.1 | |

respectively. Recently, Scudamore MacDonald [24] reported a collaborative study of HPLC using immunoaffinity column. They compared two brand immunoaffinity column and obtained that there was no significant difference between them on the recovery, R.S.D.$_R$ and R.S.D.$_r$ in wheat sample spiked 3.7 μg/kg of OTA. The collaborative study using barley, the rate of recovery was 93 ± 10% at a concentration of 5 μg/kg of OTA [23]. From an inter-laboratory study for raisins, the recovery, R.S.D.$_R$ and R.S.D.$_r$ were reported to be 72.2, 4.9 and 14%, respectively [31]. In this study, the recoveries, R.S.D.$_R$ and R.S.D.$_r$ were the same level as these obtained from other collaborative studies in cereals (wheat and corn) and raisins. Vargas and Santos [25] reported the collaborative study using green coffee beans, the recovery rates from samples spiked with 4.48 μg/kg of OTA was 92.8%. The reason why the recovery of OTA in green coffee in this study was lower than that in their study is considered that the caffeine in coffee affects the immuno-reaction because the dilution rate of extract applied to immunoaffinity column used in this study was half of that used in their study.

### 3.2. Analytical results using a multifunctional clean-up column

The clean-up procedure with the multifunctional column did not give a plane baseline in the chromatogram of any materials used in this study. The presence of numerous peaks disrupted

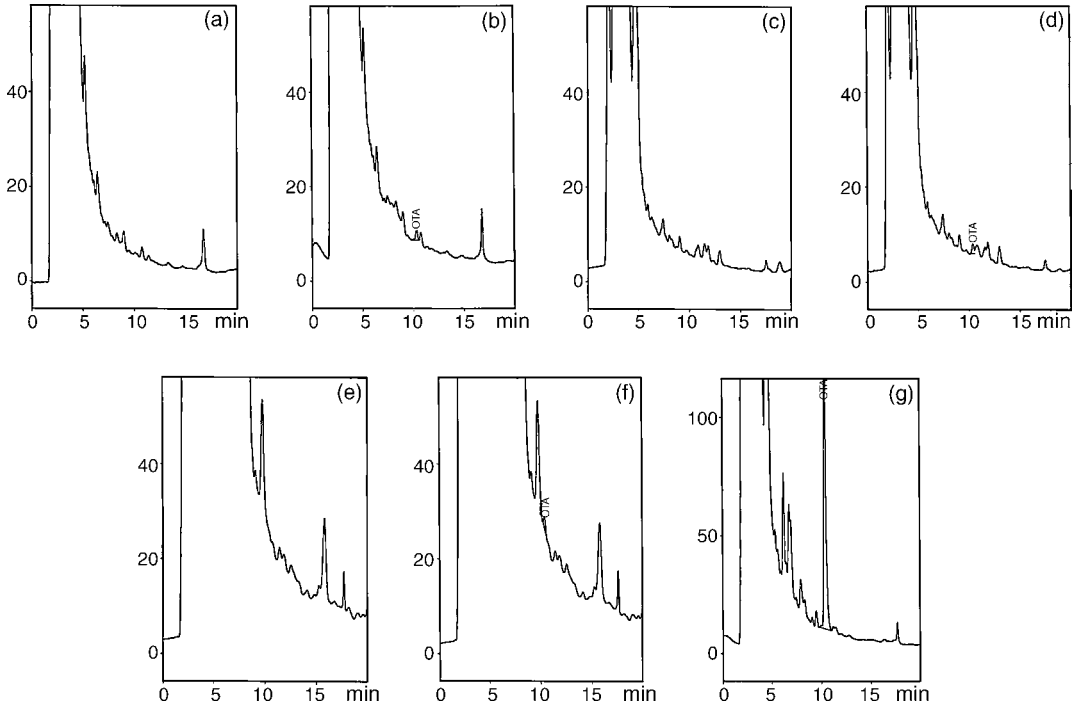


Fig. 2. Chromatograms of OTA detected in food samples using a multifunctional clean-up column: (a) blank wheat, (b) wheat spiked with 0.5 μg/kg of OTA, (c) blank corn, (d) corn spiked with 0.5 μg/kg of OTA, (e) blank green coffee beans, (f) green coffee beans spiked with 0.5 μg/kg of OTA, (g) naturally contaminated raisins. For chromatographic conditions see text.

Table 3
Results of an interlaboratory study using a multifunctional clean-up column for detection of OTA

| Number of laboratory | Spiked wheat | | | | Spiked corn | | | | Spiked green coffee beans | | | | Naturally contaminated raisins | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0.5 μg/kg | | 5.0 μg/kg | | 0.5 μg/kg | | 5.0 μg/kg | | 0.5 μg/kg | | 5.0 μg/kg | | | |
| 1 | 84.0 | 84.0 | 92.4 | 88.0 | 86.0 | 84.0 | 81.6 | 82.6 | 186.0 | 164.0 | 101.0 | 101.2 | 17.75 | 17.68 |
| 2 | 96.0 | 96.0 | 90.4 | 91.8 | 80.0 | 74.0 | 82.4 | 82.8 | 346.0 | 348.0 | 112.0 | 113.2 | 16.24 | 16.66 |
| 3 | 79.8 | 83.2 | 90.1 | 91.6 | 89.6 | 88.2 | 78.5 | 78.2 | 129.8 | 158.2 | 92.7 | 99.6 | 17.23 | 17.40 |
| 4 | 94.0 | 98.0 | 92.2 | 90.6 | 66.0 | 66.0 | 79.2 | 79.0 | N.I. | N.I. | 92.2 | 89.4 | 16.31 | 16.21 |
| 5 | 108.0 | 106.0 | 104.0 | 102.4 | 96.0 | 91.0 | 91.2 | 93.0 | N.I. | N.I. | 82.0 | 92.4 | N.I. | N.I. |
| 6 | 70.0 | 68.0 | 92.0 | 90.8 | 76.2 | 72.0 | 81.8 | 81.8 | 74.0 | 74.0 | 95.2 | 91.8 | 17.14 | 17.34 |
| Mean value (μg/kg) | | | | | | | | | | | | | 17.0 | |
| Mean recovery (%) | 88.9 | | 93.0 | | 80.8 | | 82.6 | | 185.0 | | 97.0 | | 128.7 | |
| Precision (%) | | | | | | | | | | | | | | |
| $S_r$ | 1.7 | | 1.6 | | 2.7 | | 0.6 | | 12.7 | | 3.8 | | 0.2 | |
| R.S.D.$_r$ | 1.9 | | 1.7 | | 3.3 | | 0.7 | | 6.9 | | 3.9 | | 1.1 | |
| $S_R$ | 13.5 | | 22.4 | | 19.8 | | 18.1 | | 53.2 | | 22.8 | | 0.6 | |
| R.S.D.$_R$ | 15.2 | | 24.1 | | 24.5 | | 21.9 | | 33.9 | | 23.3 | | 3.7 | |

N.I.: the peak correspoding to OTA was not identified.

identification of the peak corresponding to OTA (Fig. 2). However, the mean recoveries of all spiked samples except for the green coffee beans spiked with 0.5 μg/kg of OTA were more than 80.0%. In naturally contaminated raisins, one laboratory could not identify OTA because of the many peaks. The mean concentration of OTA in five laboratories was 17.0 μg/kg, which gave a rate of recovery of 128.7% based on the value assigned by the FAPAS report (Table 3).

The recovery, R.S.D.$_r$ and R.S.D.$_R$ values of wheat were 88.9–93.0, 1.7–1.9 and 15.2–24.1%, respectively. For corn, the recovery was 80.8%, the R.S.D.$_r$ was 3.3% and the R.S.D.$_R$ was 24.5% in the sample spiked with 0.5 μg/kg, and the recovery was 82.6%, the R.S.D.$_r$ was 0.7% and R.S.D.$_R$ was 21.9% in the sample spiked with 5.0 μg/kg of OTA. For green coffee beans, in the sample spiked with the low concentration, OTA could not be identified in two laboratories owing to the large number of peaks and the recovery was 185.0%. However, in the sample spiked with high concentration of OTA, all participants recognized OTA in the chromatogram and the recovery, R.S.D.$_r$ and R.S.D.$_R$ were 97.0, 3.9 and 23.3%. The recovery, R.S.D.$_r$ and R.S.D.$_R$ values for naturally contaminated raisins in five laboratories were 125.9, 1.1 and 3.7%, respectively. Based on *S/N* value of 3/1, the limit of determination was estimated at 1.5–2.0 μg/kg for wheat and corn. Since the clean-up using a multifunctional column is a new procedure, there is little information about the application to commodities. Buttinger et al. [26] who developed this column, reported that this procedure could be used to clean-up cereals, red wines, raisins and green coffee. Our results, indicate that it is difficult to clean-up green coffee and raisins using a multifunctional column at a level of 0.5 μg/kg.

## 4. Conclusions

More than 10 countries have proposed or enacted regulations for OTA. There is much variation in the level of tolerance among countries. The EU has set the tolerable level in raw cereals at 5 μg/kg, while the USA has proposed a level for cereals of 20 μg/kg. Generally, surveillance requires a more sensitive method of detection than enforcement does.

We compared the performances of an immunoaffinity column and a multifunctional column. The immunoaffinity column is complicated but could be used to clean-up cereals, green coffee and raisins at a low concentration of OTA. Although the use of a multifunctional column is simple and rapid, it could not be applied to use for green coffee and raisins at a low concentration of OTA. These results suggest that one has to choose a suitable clean-up procedure depending on the purpose and material.

## Acknowledgement

The authors acknowledge the financial support from a grant for Food Hygienic Research, the Japanese Ministry of Health, Labour and Welfare.

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