Occurrence of ochratoxin A and ochratoxin B in commercial coffee in Vietnam and Thailand

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Abstract

Occurrence of ochratoxin A (OTA) and ochratoxin B (OTB) in 32 Vietnamese roasted coffees, 30 Thai roasted coffees and 38 Thai instant coffees was performed using an immunoaffinity column-HPLC method. Twenty-six of Vietnamese roasted coffees were contaminated with overall average 0.75 µg/kg of OTA and 11 samples were contaminated with overall average 0.20 µg/kg of OTB. Four of Thai roasted coffees were contaminated with overall average 0.17 µg/kg of OTA and one sample was contaminated with 0.56 µg/kg of OTB. Twenty-eight of Thai instant coffees were contaminated with overall average 2.19 µg/kg of OTA, and OTB from these samples was not detected. There was no sample more than OTA regulatory limits of European Union. Therefore, it seems that the risk of ochratoxins in retail coffees in Vietnam and Thailand was acceptably low. This report is the first on occurrence of OTB in Vietnamese roasted coffee and ochratoxins in Thai instant coffee.

Ochratoxins, which have nephrotoxic, teratogenic, genotoxic and neurotoxic effects on animals and humans¹, are produced principally by several species of *Aspergillus* and *Penicillium verrucosum*². Ochratoxins occur in various cereals and beans and their related products, wine, meats and meat products³. Ochratoxin A (OTA), which is classified as a possible human carcinogen (Group 2B) by the International Agency for Research on Cancer², is the most potent ochratoxin. Ochratoxin B (OTB), which is the non-chlorinated analog of OTA, was lower toxic than OTA⁴. The lower levels of OTB than OTA has been found in cereals, cacao and coffee products in Japan^{5,6}. However, red wines in Italy and Spain were contaminated with OTB which was almost the same levels of OTA^{7,8}. Because 22 (28.6%) of the 77 red wines in these reports were contaminated with higher concentration of OTB than OTA, we considered that it was preferable to analyze both OTA and OTB in coffee products.

Coffee has been found to be contaminated with OTA at a relatively high frequency⁹. In the European Union, the OTA regulatory limit is 5 µg/kg for roasted coffee and 10 µg/kg for instant coffee¹⁰, but a regulatory limit for OTA in food has not been established in Thailand and Vietnam¹¹. Vietnam is the world's second largest coffee producer, mainly of Robusta coffee. Its Robusta coffee is exported around the world and com-

monly consumed as a regular coffee in Vietnam¹². Thailand is Southeast Asia's third largest coffee exporter¹². In Thailand, Arabica beans are cultivated in the mountains at high altitude in the north, whereas Robusta beans are cultivated in the south¹³. There are some reports on contamination of OTA and infection of OTA-producing fungi in green coffee in Vietnam^{14,15} and Thailand¹³. Only two reports on OTA in Vietnamese roasted coffee and Thai roasted coffee have been published^{16,17}, but there are no reports in international journals on ochratoxins in Thai instant coffee. The aim of this study was to survey OTA and OTB in commercial coffee from Vietnam and Thailand using an in-house immunoaffinity-column (IAC) HPLC method⁶.

Thirty-two roasted coffees (Robusta) were purchased from conventional supermarkets and stores in Ho Chi Minh City and Can Tho City in Vietnam during 2011 and 2012. Thirty roasted coffees (Arabica) were purchased in Chiang Mai City and 38 instant coffees (mainly Robusta) were purchased in Chiang Mai and Bangkok in Thailand during 2012 and 2013. All samples were kept at 4°C until analysis. Acetonitrile and methanol (HPLC-grade), and other chemicals (reagent grade) were purchased from Wako Pure Chemicals (Osaka, Japan). Water was purified using a Milli-Q ultra-purification system (Millipore, Bedford, MA, USA). Standard solutions of OTA and OTB (10 µg/mL in acetonitrile)

were purchased from Sigma-Aldrich (St. Louis, USA). Equal volumes of the standard solutions were mixed and 1 µg each of OTA and OTB were dispensed into a test tube, evaporated to dryness, and stored at −20°C until used.

Roasted coffee analysis was carried out using a small modified method of Kawamura *et al.*^[6]. Finely ground roasted coffee of 8.0 g was placed on filter paper. OTA and OTB were extracted with 140 mL of hot water by using a commercially available coffee making machine (TSK-117; EUPA International Corporation, Pasadena, United States). The extracted coffee (36 mL) was added to 4 mL of 5% NaHCO₃, and then filtered with a glass fiber filter (GS-25; Advantec, Tokyo, Japan). Ten mL of the filtrate was applied to the in-house IAC.

Instant coffee analysis was carried out using a modified method of Nakajima *et al.*^[18]. Instant coffee of 5.0 g was added to 80 mL of 1% NaHCO₃ solution, sonicated for 15 min, and mixed for 30 min using an almighty shaker (AS ONE, Osaka, Japan). Then, the resultant instant coffee extract (ca. 80 mL) was adjusted to 100 mL with 1% NaHCO₃. After centrifuging at 3,000 rpm for 20 min, the upper layer was collected and filtered using the glass fiber filter. A 20 mL of filtrate was diluted with 20 mL PBS, a 10 mL of the diluted filtrate was applied to the IAC.

The purified antibody named OTB.2, which was bispecific to OTA and OTB^[6], was coupled to affinity chromatography resin (Affi-Gel 10; Bio-Rad, Hercules, United States) in the ratio of 4 mg of antibody per 1 mL of the resin. The resin containing the antibody (0.3 mL) was packed into a mini column (Muromac column size S; Muromachi Technos Co., Ltd., Tokyo, Japan). The in-house IAC was conditioned with 10 mL of Dulbecco's phosphate buffered saline (PBS, 136.9 mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, and 8.1 mM Na₂HPO₄ · 12H₂O). Sample solution (10 mL) was applied to the IAC at a flow rate of below 1 mL/min. The IAC was then washed with 10 mL of PBS to remove interferents. OTA and OTB were eluted with 3 mL of methanol and the eluate was evaporated to dryness. The sample solution for HPLC analysis was prepared dissolving the residue in 0.5 mL of acetonitrile:water (40+60, v/v).

HPLC system (Shimadzu, Kyoto, Japan) was LC-10ADVP pump, SIL-10ADVP autosampler, CTO-10AXL column oven, and RF-10ADXL SUPER fluorescence detector. Chromatographic separation was performed using a Shiseido Capcell Pak C18 MG (5.0 µm particle size, 4.6 × 250 mm; Shiseido, Tokyo, Japan) at 40°C. The mobile phase was acetonitrile:water:acetic acid (40+58+2, v/v/v) set at a flow rate of 1.0 mL/min. Injection volume was 20 µL. The fluorescence detector was used with an excitation wavelength of 335 nm and emission wavelength of 465 nm. Seven concentrations of OTA and OTB standards (0.16, 0.31, 0.63, 1.25, 2.5, 5.0 and 10.0 µg/L) in acetonitrile:water (40+60, v/v) were prepared to determine linearity. The calibration curves were linear with a correlation coefficient (R^2) 0.999 for

both OTA and OTB. The HPLC chromatogram of the OTA and OTB standard solutions (2.5 µg/L) is shown in Fig. 1 A. Based on a signal/noise ratio of 10, the limits of quantification (LOQ) for OTA and OTB were 0.2 µg/kg in the roasted and 0.4 µg/kg in instant coffee, respectively.

The OTA and OTB values in 32 Vietnamese and 30 Thai roasted coffees, and 38 Thai instant coffees were shown in Table 1, 2 and 3, respectively, with associated chromatograms of Fig. 1 B, 1 C and 1 D. The OTA values in these coffee samples were lower than the regulatory limit (5 µg/kg for roasted coffee and 10 µg/kg for instant coffee) in the European Union.

For Vietnamese roasted coffee samples, the 26 (81.3%) of the 32 samples were contaminated with OTA with a positive average of 0.90 µg/kg in the range 0.24–4.27 µg/kg (Table 1). The overall average (samples < LOQ were given the LOQ/2 value (0.1 µg/kg)) was 0.75 µg/kg. Additionally, the 11 (34.4%) of the 32 samples were contaminated with OTB in the range 0.21–0.64

Fig. 1 HPLC chromatograms of ochratoxin A and B standards and naturally-contaminated roasted and instant coffee samples.

- A) A mixture of ochratoxin A and B standards (each 2.5 µg/L).
- B) Vietnamese roasted coffee (VC120304) contaminated with 4.27 µg/kg of ochratoxin A and 0.64 µg/kg of ochratoxin B.
- C) Thai roasted coffee (RCT12036) contaminated with 0.52 µg/kg of ochratoxin A.
- D) Thai instant coffee (ICT1303) contaminated with 4.68 µg/kg of ochratoxin A.

OTA: ochratoxin A, OTB: ochratoxin B

Table 1 Ochratoxin A and B in Vietnamese roasted coffee

Sample	OTA ^a (µg/kg)	OTB ^b (µg/kg)	Sample	OTA (µg/kg)	OTB (µg/kg)
VC120301	0.75	- ^c	VC120317	0.49	-
VC120302	0.44	-	VC120318	0.97	-
VC120303	0.89	0.30	VC120319	-	-
VC120304	4.27	0.64	VC120320	-	-
VC120305	0.42	-	VC120201	0.84	0.38
VC120306	0.47	-	VC120202	0.61	0.21
VC120307	0.31	-	VC120203	0.67	-
VC120308	0.53	-	VC120204	0.52	-
VC120309	1.81	0.27	VC120205	0.58	0.37
VC120310	0.43	-	VC120206	-	-
VC120311	0.40	-	VC120207	0.25	-
VC120312	0.41	-	VC120208	-	-
VC120313	-	-	VC120209	1.17	0.42
VC120314	0.24	-	VC120210	2.34	0.52
VC120315	-	-	VC120211	1.65	0.47
VC120316	1.03	0.25	VC120212	0.93	0.58
Positives average	0.90	0.40	Overall average ^d	0.75	0.20
Japanese roasted coffee positives average ⁶⁾	0.18	0.07	Japanese roasted coffee overall average ⁶⁾	0.11	0.02

^a Ochratoxin A^b Ochratoxin B^c < LOQ (0.2 µg/kg) for both ochratoxin A and B.^d Samples less than LOQ (0.2 µg/kg) were given the LOQ/2 value (0.1 µg/kg).**Table 2** Ochratoxin A and B in Thai roasted coffee

Sample	OTA ^a (µg/kg)	OTB ^b (µg/kg)	Sample	OTA (µg/kg)	OTB (µg/kg)
RCT12011	- ^c	-	RCT12028	-	-
RCT12012	-	-	RCT12029	0.70	-
RCT12013	-	-	RCT12030	-	-
RCT12014	-	-	RCT12031	-	-
RCT12015	-	-	RCT12032	-	-
RCT12016	-	0.56	RCT12033	-	-
RCT12017	-	-	RCT12034	-	-
RCT12018	-	-	RCT12035	-	-
RCT12019	-	-	RCT12036	0.52	-
RCT12020	-	-	RCT12037	-	-
RCT12021	1.00	-	RCT12038	-	-
RCT12022	-	-	RCT12039	0.40	-
RCT12023	-	-	RCT12040	-	-
RCT12024	-	-	RCT12041	-	-
RCT12025	-	-	RCT12042	-	-
Positives average	0.66	0.56	Overall average ^d	0.17	0.12
Japanese roasted coffee positives average ⁶⁾	0.18	0.07	Japanese roasted coffee overall average ⁶⁾	0.11	0.02

^a Ochratoxin A^b Ochratoxin B^c < LOQ (0.2 µg/kg) for both ochratoxin A and B.^d Samples less than LOQ (0.2 µg/kg) were given the LOQ/2 value (0.1 µg/kg).

Table 3 Ochratoxin A and B in Thai instant coffee

Sample	OTA ^a (µg/kg)	OTB ^b (µg/kg)	Sample	OTA (µg/kg)	OTB (µg/kg)
ICT1301	3.01	- ^c	ICT1320	3.03	-
ICT1302	4.52	-	ICT1321	2.81	-
ICT1303	4.68	-	ICT1322 ^e	1.57	-
ICT1304 ^e	1.40	-	ICT1323	-	-
ICT1305	2.62	-	ICT1324	-	-
ICT1306	1.28	-	ICT1325	-	-
ICT1307	2.62	-	ICT1326	2.58	-
ICT1308	2.08	-	ICT1327	2.36	-
ICT1309	3.90	-	ICT1328	-	-
ICT1310	2.97	-	ICT1329	2.55	-
ICT1311	2.18	-	ICT1330	1.43	-
ICT1312	6.20	-	ICT1331	-	-
ICT1313	-	-	ICT1332	-	-
ICT1314 ^e	3.12	-	ICT1333	4.34	-
ICT1315	2.08	-	ICT1334	3.76	-
ICT1316	3.48	-	ICT1335	-	-
ICT1317	3.74	-	ICT1336	1.60	-
ICT1318	2.85	-	ICT1337	-	-
ICT1319	2.50	-	ICT1338	-	-
Positives average	2.90	-	Overall average ^d	2.19	0.2
Japanese instant coffee positives average ⁶⁾	0.46	0.22	Japanese instant coffee overall average ⁶⁾	0.35	0.06

^a Ochratoxin A^b Ochratoxin B^c < LOQ (0.4 µg/kg) for both ochratoxin A and B.^d Samples less than LOQ (0.4 µg/kg) were given the LOQ/2 value (0.2 µg/kg).^e ICT 1304, ICT 1314 and ICT 1322 were imported from Brazil.

µg/kg. The positive average was 0.40 µg/kg. The positive ratio of OTA was 2.4-times higher than that of OTB. The average concentration of OTA was 2.3-times higher than that of OTB. The finding was that the samples with OTA that larger than 0.6 µg/kg tended to contain OTB (Table 1). In the only published study of OTA in Vietnamese roasted coffee, Napolitano *et al.*¹⁶⁾ reported that 1.5 µg/kg of OTA were found. The positive average of OTA in Vietnamese roasted coffee in this study was similar to this previous report. The 549 (46.3%) of the 1,184 roasted coffees in the European Union were contaminated with OTA with an overall average of 0.72 µg/kg³⁾. The 18 (36.7%) of the 49 retail roasted coffees in Japan (2004–2007) were contaminated with OTA with a positive average of 0.55 µg/kg and an overall average of 0.20 µg/kg¹⁹⁾. The positive ratio of OTA in Vietnamese roasted coffee were 1.8-times higher than that in the European Union and 2.2-times higher than that in Japan. The overall average of OTA in Vietnamese roasted coffee was almost the same as the European Union. The positive average of OTA in Vietnamese roasted coffee was about 2-times higher than that in Japan. In one of the most recent reports of OTA and

OTB in Japanese roasted coffees, the 30 (62.5%) of the 48 samples were contaminated with OTA with a positive average of 0.18 µg/kg⁶⁾. OTB was detected in 37.5% of the samples with a positive average of 0.07 µg/kg⁶⁾ (Table 1). The positive average of OTA in Vietnamese roasted coffee was 5-times, and the average of OTB in Vietnamese roasted coffee was about 6-times higher than this recent report in Japan⁶⁾.

For Thai roasted coffee samples, only 4 (13.3%) of the 30 samples were contaminated with OTA with an average of 0.66 µg/kg in the range 0.40–1.00 µg/kg (Table 2). The overall average was 0.17 µg/kg. OTB was detected in only one sample (0.56 µg/kg). Chompurat *et al.* reported that 3 (6.8%) of 44 Thai roasted coffee (Arabica-Robusta blended) were contaminated with OTA with a positive average of 2.9 µg/kg in the range <1–4.6 µg/kg¹⁷⁾. The positive average of OTA in the report was 4.3-times higher than that in this study. In other reports, 46.3% of roasted coffees (overall average of 0.72 µg/kg) in the European Union³⁾ and 36.7% of roasted coffees (overall average of 0.20 µg/kg) in Japan¹⁹⁾ were contaminated with OTA. The incidence of OTA in Thai roasted coffee was 2.8-times lower than in

Japan and 3.5-times lower than the European Union. The overall average of OTA in our samples was 4.2-times lower than the European Union and almost the same as the data from Japan. In the recent report in Japan, 62.5% of roasted coffees (positives average of 0.18 µg/kg)⁶⁾ were contaminated with OTA (Table 2). The positive average of OTA in Thai roasted coffee was 3.7-times higher than this recent report in Japan⁶⁾. The overall average of Thai roasted coffee (Arabica) was 4.4-times lower than the Vietnamese roasted coffee (Robusta) in this study. Noonim *et al.* reported that OTA in the Robusta green coffee was 1.6-times higher than the Arabica green coffee in Thailand¹³⁾. Obanos *et al.* reported that Robusta green coffee from Vietnam, India and Uganda were contaminated with 30-times higher OTA than Arabica green coffee from Colombia and Costa Rica and 9-times higher OTA than Arabica green coffee from Brazil¹⁴⁾. These reports support our results that Robusta coffee were contaminated with higher OTA than Arabica coffee.

For Thai instant coffee samples, the 28 (73.7%) of the 38 samples were contaminated with OTA with a positive average of 2.90 µg/kg in the range 1.28–6.20 µg/kg (Table 3). The overall average was 2.19 µg/kg. The OTB in these samples were not detected. Noonim *et al.* isolated *Aspergillus carbonarius* from Robusta dried cherries and green coffees in Thailand, and the isolated *A. carbonarius* produced OTA, but not OTB¹³⁾. From the report, we estimated the OTA producing-fungi in the instant coffee to be *A. carbonarius*. The 63 (95.5%) of the 66 retail instant coffees in Japan (2005–2007) were contaminated with OTA with a positive average of 0.72 µg/kg¹⁹⁾. In the recent report, the 22 (73.3%) of the 30 Japanese instant coffees were contaminated with OTA with a positive average of 0.46 µg/kg⁶⁾ (Table 3). Nielsen *et al.* reported that 14 (56%) of the 25 retail instant coffees in Denmark, Kenya, Tanzania, and Uganda were contaminated with OTA with a positive average of 4.5 µg/kg²⁰⁾. The average of OTA in Thai instant coffees was about 4- to 6-times higher than Japan, but about 2-times lower than Denmark, Kenya, Tanzania and Uganda.

In total, we analyzed OTA and OTB in 100 commercial coffee samples from Vietnam and Thailand. The 59 (59%) of the 100 samples were contaminated with OTA or OTB, and the OTA values in analyzed coffee samples were lower than the regulatory limit in the European Union. The coffee samples in this study were coffee products sold in market for only a year. The level of OTA contamination in coffee products will vary according to the background that coffee was produced. It is important to perform the monitoring or survey of OTA and OTB for commercial coffee products in Vietnam and Thailand continually, although the level was acceptable in this study. We reported the occurrence of OTB in commercial Vietnamese roasted coffee and ochratoxins in Thai instant coffee for the first time.

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