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ARTICLE

# Determination of Ochratoxin A in Ready-To-Drink Coffee by Immunoaffinity Cleanup and Liquid Chromatography−Tandem Mass Spectrometry

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We developed a simple and accurate method for determining ochratoxin A (OTA) in ready-to-drink coffee, using an immunoaffinity column for cleanup and liquid chromatography−tandem mass spectrometry (LC/MS/MS) for identification and quantification. When uniformly stable isotope-labeled OTA (U-[$^{13}C_{20}$]-OTA) was employed as an internal standard, the recovery rate of the method was 97.3% (the spiked OTA level was 0.10 ng/mL), the repeatability (relative standard deviation) was 1.9%, and the intermediate precision (relative standard deviation) was 4.0%. The limit of quantification was 0.0065 ng/mL based on a signal-to-noise ratio in coffee of 10:1. The developed method was used for the determination of OTA in ready-to-drink coffee. A total of 30 ready-to-drink coffee samples commercially available in Japan were analyzed. OTA was detected in all of the samples at concentrations ranging from trace levels (0.0020−0.010 ng/mL) to 0.037 ng/mL. This method was shown to be useful for accurately evaluating the intake of OTA from coffee beverages.

**KEYWORDS: Ochratoxin A; mycotoxins; coffee; LC/MS/MS**

## INTRODUCTION

Ochratoxin A (OTA; **Figure 1**) is a widely distributed mycotoxin that is produced by several species of fungi in the genera *Aspergillus* and *Penicillium* (*1*). These fungi can grow in various climates (*Aspergillus* in warm and tropical regions and *Penicillium* in temperate regions); hence, the contamination of food crops with OTA has been identified as a food-safety issue worldwide (*2*). Because OTA is reported to be nephrotoxic and carcinogenic to both humans and animals and the International Agency for Research on Cancer (IARC) has classified it as belonging to Group 2B ("possibly carcinogenic to humans") (*3*). The health risk posed by OTA has been assessed in various studies, and a provisional tolerable weekly intake level of 100 ng/kg body weight (bw) has been established by the Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives (JECFA) (*4*).

OTA is commonly found in foods and beverages such as cereals, beans, dried fruits, beer, wine, and coffee (*5*). The presence of OTA in coffee beans has been documented since 1974 (*6*−*12*). In fact, the JECFA reported that coffee accounted for around 6% of the total OTA intake in humans in 2001 (*4*). Several countries have their own regulations regarding the OTA content of coffee; for instance, the maximum level in coffee beans permitted by the European Union is 5.0 μg/kg (*13*). OTA has therefore become of great importance to the coffee industry worldwide. In Japan, regulatory limits for OTA have not yet been instituted; however, in 2005, the Ministry of Health, Labor, and Welfare of Japan investigated its occurrence in retail foods, including 20 types of coffee bean, to evaluate the health risk to consumers (*14*). In view of these considerations, there is an urgent need for sensitive and accurate methods to determine the OTA level in coffee.

In previous studies, the OTA contents of coffee and green and roasted coffee beans have generally been analyzed using organic solvents for extraction. The transfer of OTA from the beans into the beverages during coffee brewing has also been investigated, and the results have ranged from 0 to nearly 100% (*15*−*18*). This inconsistency is probably due to differences in the processing conditions, such as the roasting levels. A reduction of the OTA level has been reported to occur during the roasting and brewing processes in coffee manufacture (*19*, *20*); therefore, the daily intake of OTA from coffee can be assessed more accurately by analyzing coffee beverages than by investigating natural coffee bean contamination.

Bottled and canned ready-to-drink coffees are popular in Japan, and various types are commercially available (*21*). The total production volume of ready-to-drink coffees is about 3 million kL/year and exceeds that of bottled mineral waters. We therefore aimed to analyze the commercially available ready-to-drink coffees, to determine the OTA intake of Japanese consumers.

The objective of the current study was to establish a simple and accurate method for OTA determination by removing the complex matrices, which include various additives, such as milk and sugar. To achieve high sensitivity and selectivity, immunoaffinity

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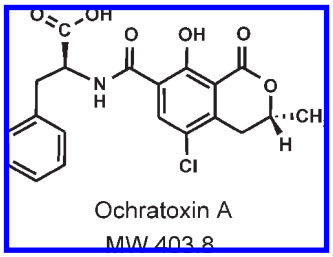


**Figure 1.** Structure and molecular weight (MW) of OTA.

cleanup and liquid chromatography−tandem mass spectrometry (LC/MS/MS) were used (*22*). Additionally, the efficacy of uniformly stable isotope-labeled OTA (U-[$^{13}C_{20}$]-OTA) as an internal standard was evaluated. The method developed here was applied to Japanese ready-to-drink coffees to investigate their OTA contents.

## MATERIALS AND METHODS

**Sampling.** Samples (190−280 mL) of 25 canned and 5 bottled ready-to-drink coffees were obtained in a random manner from local supermarkets and small retail outlets in Japan during 2007. All of the coffee samples were Japanese brands made in Japan and stored at 4 °C until analysis.

**Chemicals and Materials.** The OTA standard (50 μg/mL) was purchased from Sigma-Aldrich (Tokyo, Japan). The U-[$^{13}C_{20}$]-OTA standard (10 μg/mL) was purchased from biopure (Tulln, Austria). The OTA stock solutions (2 μg/mL) were prepared by diluting the standards in toluene/acetic acid [99:1 (v/v)] and were stored at 4 °C. The stock solutions were vortexed and equilibrated at room temperature before use. The calibration standard solutions for LC/MS/MS were prepared by diluting the stock solution in water/acetonitrile/acetic acid [70:30:1 (v/v/v)]. Acetonitrile (LC/MS grade), methanol (LC/MS grade), 0.1 vol % formic acid−distilled water [high-performance liquid chromatography (HPLC) grade], 0.1 vol % formic acid−acetonitrile (HPLC grade), toluene [pesticide residue and polychlorinated biphenyl (PCB) analysis grade], sodium hydrogen carbonate (analytical grade), ammonium acetate (analytical grade), sodium chloride (pesticide residue and PCB analysis grade), sodium dihydrogenphosphate dehydrate (analytical grade), and disodium hydrogenphosphate 12-water (analytical grade) were obtained from Kanto Chemical Co., Inc. (Tokyo, Japan). Acetic acid (LC/MS grade) was obtained from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). OchraTest immunoaffinity columns were obtained from Vicam (Watertown, MA). Polytetrafluoroethylene (PTFE) syringe filters (0.45 μm) were obtained from Advantec (Tokyo, Japan).

**Sample Preparation for Ready-To-Drink Coffees.** The U-[$^{13}C_{20}$]-OTA standard solution was added at a concentration of 0.10 ng/mL to each sample as an internal standard before the immunoaffinity column cleanup. Each 5 mL ready-to-drink coffee sample was diluted with 40 mL of 100 mM phosphate buffer at pH 8.0. The diluted extract was cleaned by passing it through an OchraTest immunoaffinity column at a flow rate of 1 drop/s. The column was washed with 6 mL of aqueous solution containing sodium chloride (2.5%) and sodium hydrogen carbonate (0.5%), followed by 6 mL of aqueous solution containing ammonium acetate, and then air-dried. The OTA was eluted with 3 mL of methanol/acetic acid (98:2 [v/v]) and collected in a test tube. The eluted extract was evaporated to dryness under a nitrogen stream at 40 °C and dissolved with 1 mL of water/acetonitrile/acetic acid (70:30:1 [v/v/v]). Each sample was filtered on a 0.45 μm PTFE filter immediately before the LC/MS/MS analysis.

**Separation and Quantification.** The OTA analysis was carried out using an Agilent 1100 series HPLC system (Agilent Technologies, Santa Clara, CA) coupled with a triple-stage quadrupole API4000 MS/MS system (Applied Biosystems, Foster City, CA).

Analyst 1.4.1 software was used to control the instruments and process the data. The analytical columns employed were a XBridge C18 column (2.1 mm inner diameter × 100 mm, 3.5 μm; Waters, Milford, MA) and a XBridge C18 guard column (2.1 mm inner diameter × 10 mm, 3.5 μm) operated at 40 °C. The mobile phase consisted of (A) 0.1 vol % formic acid−water and (B) 0.1 vol % formic acid−acetonitrile. A linear gradient profile with the following proportions (v/v) of solvent (B) was applied at a flow rate of 0.2 mL/min: [*t* (min), % B]: (0, 30), (1, 30), (11, 90), and (16, 90). The total run time, including the conditioning of the column to the initial conditions, was 25 min. An auto sampler was used for injection at a volume of 10 μL. Under these conditions, the retention time was typically 10.4 min for both OTA and U-[$^{13}C_{20}$]-OTA.

**Table 1.** LC/MS/MS Conditions for Selected Parameters

| *m/z* | DP (V) | CE (V) | CXP (V) |
|---|---|---|---|
| 404 → 239 | 66 | 33 | 14 |
| 404 → 358 | 66 | 21 | 18 |
| 404 → 221 | 66 | 47 | 18 |
| 424 → 250 | 66 | 33 | 14 |
| 424 → 377 | 66 | 21 | 20 |
| 424 → 232 | 66 | 49 | 12 |

The electrospray-ionization (ESI) source was operated in the positive-ion mode. The operating parameters were optimized by a flow-injection methodology (FIA) as follows: curtain gas, 10 psi; ion source gas 1, 40 psi; ion source gas 2, 60 psi; ionspray voltage, +5500 V; collision gas, 5 (arbitrary units); and temperature, 600 °C. Data acquisition for quantification and confirmation was performed by working in the multiple-reaction monitoring (MRM) mode. The following precursor-to-fragment transitions were used for quantification: *m/z* 404 → 239 (OTA) and *m/z* 424 → 250 (U-[$^{13}C_{20}$]-OTA) with a dwell time of 200 ms; and for confirmation, *m/z* 404 → 358 (OTA), *m/z* 404 → 221 (OTA), *m/z* 424 → 377 (U-[$^{13}C_{20}$]-OTA), and *m/z* 424 → 232 (U-[$^{13}C_{20}$]-OTA) with a dwell time of 100 ms. The parameters used for the OTA analysis were optimized in an infusion experiment. The selected parameters, which are shown in **Table 1**, were the declustering potential (DP), the collision energy (CE), and the collision cell-exit potential (CXP).

For quantification, calibration curves were generated from the peak−area ratio between OTA and U-[$^{13}C_{20}$]-OTA. Six calibration standards were prepared in the range of 0.050−10 ng/mL and were spiked with a constant level of internal standard (U-[$^{13}C_{20}$]-OTA). The OTA contents of the coffee samples were calculated by extrapolating the peak−area ratio to the calibration curve.

**Performance Evaluation.** The performance of the developed method was assessed using coffee samples spiked with OTA. To evaluate its efficacy for quantification, the method was compared with and without the internal standard. The coefficient of linearity was determined using coffee samples spiked with OTA at levels of 0.010, 0.050, 0.10, 0.50, 1.0, and 2.0 ng/mL. The recovery and repeatability [relative standard deviation (RSD) %] experiments were carried out using coffee samples spiked with OTA at levels of 0.01, 0.10, and 1.0 ng/mL by making six replicate measurements on the same day. The intermediate precision (RSD %) was determined using coffee samples spiked with OTA at a level of 0.10 ng/mL on 6 different days. The limit of detection (LOD) and the limit of quantification (LOQ) were determined on the basis of a signal-to-noise ratio of 3:1 for the LOD and 10:1 for the LOQ.

## RESULTS AND DISCUSSION

**Method Optimization: Cleanup Conditions.** Initially, recovery studies were performed to determine the best conditions for sample cleanup. The method described by Leoni et al. (*17*) for roasted and instant coffees was used to improve the pretreatment method for the determination of OTA in ready-to-drink coffee. This method was chosen because it involved one cleanup step using an immunoaffinity column. Before each extracted coffee sample was loaded onto the immunoaffinity column, it was diluted by an equal amount of phosphate-buffered saline (PBS), and no additional pretreatment column or liquid−liquid extraction treatments were required. Although the recoveries reported in the Leoni et al. study ranged from over 98% to almost 100% for roasted and instant coffees, they decreased to below 50% when the method was applied to a ready-to-drink coffee ($n = 2$) at a spiking level of 0.10 ng/mL OTA in the current study (**Table 2**). The low recoveries of OTA might have been attributable to

**Table 2.** Effect of Diluting Solution on the Recovery of OTA from a Coffee Sample[a]

| diluting solution | coffee[b]/diluting solution (v/v) | final pH of solution | recovery (%) |
|---|---|---|---|
| − | 1:0 | 6.2 | 35.4 |
| PBS | 1:1 | 6.7 | 49.6 |
| 100 mM phosphate buffer at pH 6 | 1:1 | 6.0 | 23.2 |
| 100 mM phosphate buffer at pH 7 | 1:1 | 6.9 | 62.3 |
| 100 mM phosphate buffer at pH 8 | 1:1 | 7.5 | 69.9 |
| 100 mM phosphate buffer at pH 9 | 1:1 | 9.0[c] | 63.2 |
| 100 mM phosphate buffer at pH 8 | 1:2 | 7.6 | 73.4 |
| 100 mM phosphate buffer at pH 8 | 1:4 | 7.8 | 79.5 |
| 100 mM phosphate buffer at pH 8 | 1:8 | 7.9 | 81.7 |
| 5% $NaHCO_3$ | 1:8 | 7.9 | 68.3 |

[a] The coffee sample contained coffee, milk, sugar, dextrin, emulsifying agent, casein sodium, flavoring, and vitamin C according to the ingredients label. [b] The spiking level was 0.10 ng/mL ($n = 2$). [c] The pH was adjusted using 1 N NaOH.

matrix interference in the ready-to-drink coffee, because it contained various additives (such as milk, sugar, and aroma chemicals) according to the ingredients label.

The recovery improved to 69.9% when the diluting solution was changed from PBS to 100 mM phosphate buffer at pH 8 with the same dilution ratio. The recoveries increased as the pH of the diluting solution increased from 6.0 to 8.0 and then decreased at pH 9.0 (this was adjusted by 1 N NaOH, because the final pH of the solution was 7.7 when 100 mM phosphate buffer at pH 9.0 was added to the coffee sample). The best results were obtained using 100 mM phosphate buffer at pH 8.0 and increasing the dilution ratio. The recoveries increased from 69.9 to 81.7% as the dilution ratio changed from 1 to 8. These findings suggested that not only the pH and the dilution ratio but also the selection of an adequate buffer contributed to the recovery, because using 5% $NaHCO_3$ as a diluting solution failed to produce a good result. Polyethylene glycol, which is known to give good recoveries for red wine (*23*), was not found to be useful in our experiment (data not shown). Additionally, the recoveries were elevated to 97.3% using U-[$^{13}C_{20}$]-OTA as an internal standard under the optimal conditions (**Table 3**).

**Method Optimization: LC/MS/MS Conditions.** OTA and U-[$^{13}C_{20}$]-OTA were detectable in the ESI positive mode in the form of $[M + H]^+$ ions. Panels **A** and **B** of **Figure 2** show the product ion spectra of the $[M + H]^+$ ions of OTA and U-[$^{13}C_{20}$]-OTA, respectively. At a collision energy of 33 eV, the major fragments formed were $[M + H-\text{phenylalanine}]^+$ ions at $m/z$ 239 and 250, which were used for quantification. Two other fragments, $[M + H-HCOOH]^+$ ions at $m/z$ 377 and 357 and $[M + H-\text{phenylalanine}-H_2O]^+$ ions at $m/z$ 232 and 221, were both selected for confirmation; their collision energies were optimized at 21 and 49 eV, respectively.

Panels **A** and **B** of **Figure 3** show typical chromatograms of a coffee sample spiked with OTA (0.10 ng/mL) and U-[$^{13}C_{20}$]-OTA (0.10 ng/mL), respectively. Because the coffee samples were concentrated 5-fold by the pretreatment, the peak intensity detected in the coffee sample spiked with 0.1 ng/mL OTA corresponded to about 0.50 ng/mL of the OTA standard. All of the coffee samples yielded good chromatograms without any interfering signals. U-[$^{13}C_{20}$]-OTA showed good performance as an internal standard, because the retention time and peak intensity were similar to those of OTA without any unwanted behaviors, such as cross talk.

**Method Performance without Internal Standard.** Results of the evaluation of the method are reported in **Table 3**. The linearity values of the standard calibration curve (0.050−10 ng/mL) and the spiked samples (0.010−2.0 ng/mL) were both $>0.999$.

**Table 3.** Performance of the Method for Determining OTA in Coffee

| | with internal standard | without internal standard |
|---|---|---|
| linearity (0.010−2.0 ng/mL) | 0.999 | 0.999 |
| recovery ($n = 6$) | | |
| 0.010 ng/mL | 99.7% | 84.4% |
| 0.10 ng/mL | 97.3% | 80.1% |
| 1.0 ng/mL | 101.6% | 83.2% |
| repeatability (RSD %, $n = 6$) | | |
| 0.010 ng/mL | 3.4% | 4.1% |
| 0.10 ng/mL | 1.9% | 2.5% |
| 1.0 ng/mL | 1.7% | 1.9% |
| LOD (S/N = 3) | 0.0020 ng/mL | 0.0020 ng/mL |
| LOQ (S/N = 10) | 0.0065 ng/mL | 0.0065 ng/mL |
| intermediate precision (RSD %, 0.10 ng/mL, $n = 6$) | 2.4% | 6.4% |

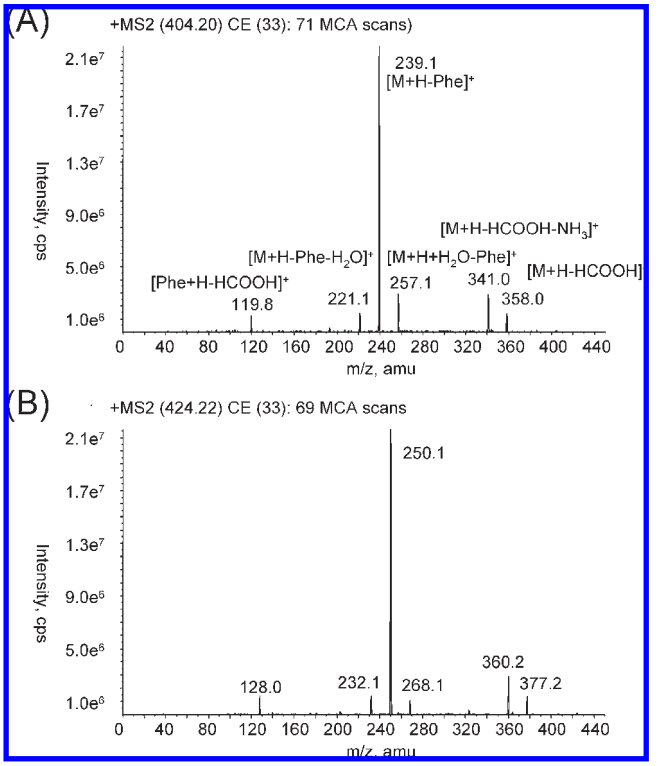


**Figure 2.** Product ion spectra of (A) OTA and (B) U-[$^{13}C_{20}$]-OTA at a collision energy of 33 eV.

The recovery rates varied from 80.1 to 84.4% within three spiked levels of OTA, and the average recovery (mean of means) was 82.6%. The loss of recovery could not be due to ion suppression, because signal reduction was not observed when we spiked OTA in the coffee matrix sample after pretreatment. The repeatability (RSD %) varied from 1.9 to 4.1% within three spiked levels of OTA, and the intermediate precision (RSD %) was 6.4%. The LOQ was 0.0065 ng/mL, and the LOD was 0.0020 ng/mL based on a signal-to-noise ratio. Chromatogram of a naturally contaminated coffee sample with a close to the LOQ level of OTA (a signal-to-noise ratio of 11) is shown in **Figure 4**. The high sensitivity was achieved by very low noise signals.

**Method Performance with Internal Standard.** To achieve better precision, the nonchlorinated analogue of OTA, ochratoxin B (OTB), has been investigated as an internal standard (*24*). However, no improvements were achieved with OTB because of differences in the chemical properties. Therefore, in the current

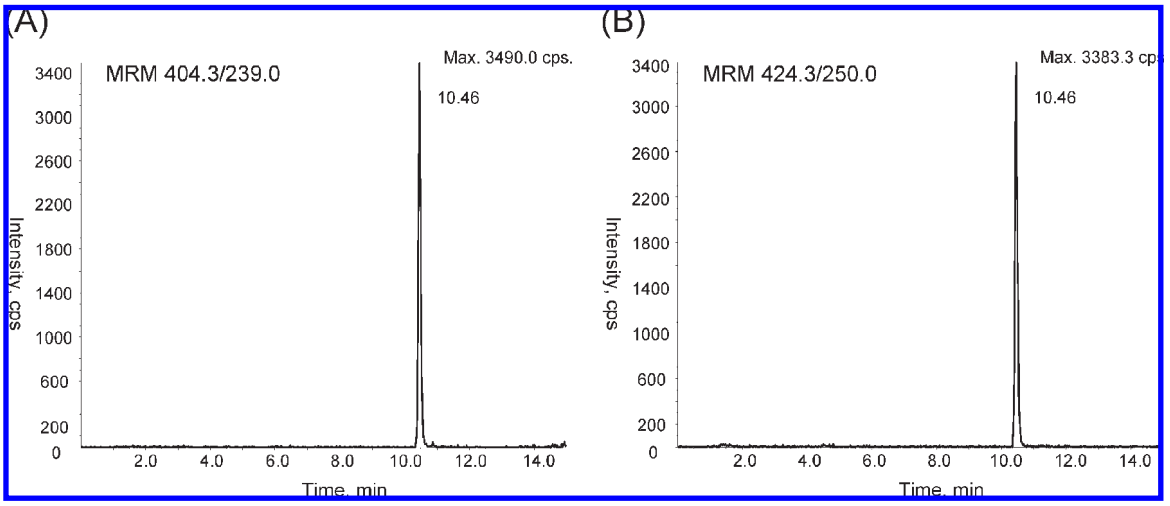


**Figure 3.** LC/MS/MS chromatograms of a coffee sample spiked with (A) OTA (0.10 ng/mL) and (B) U-[$^{13}C_{20}$]-OTA (0.10 ng/mL).

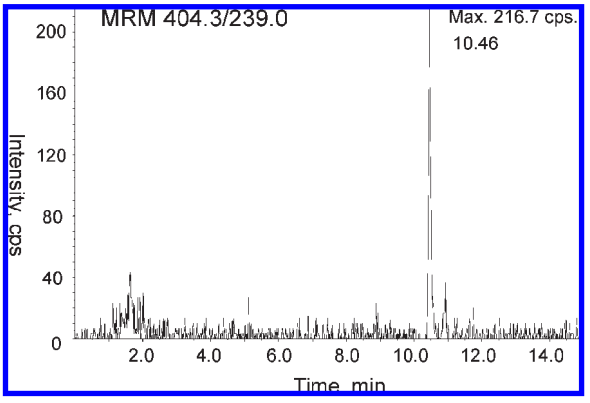


**Figure 4.** LC/MS/MS chromatogram of a naturally contaminated coffee sample with a close to the LOQ level of OTA.

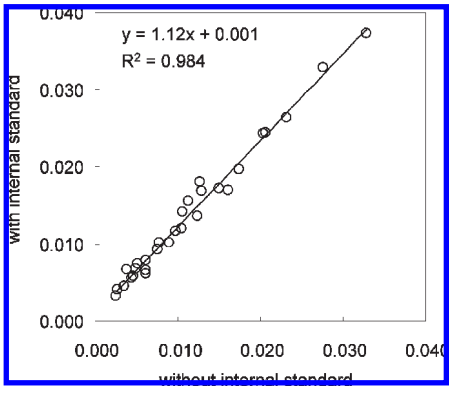


**Figure 5.** Regression curve of OTA contents (ng/mL) in 30 coffee samples with or without U-[$^{13}C_{20}$]-OTA as an internal standard.

study, we added U-[$^{13}C_{20}$]-OTA to the coffee samples before analyte extraction and investigated its efficacy as an internal standard.

The linearity values of the standard calibration curve (0.050−10 ng/mL) and the spiked samples (0.010−2.0 ng/mL) were both >0.999. The recovery rates varied from 97.3 to 101.6% within three spiked levels of OTA, and the average recovery (mean of means) was 99.5%. The repeatability (RSD %) varied from 1.7 to 3.4% within three spiked levels of OTA, and the intermediate precision (RSD %) was 2.4%. Significant improvements of the recovery rate and the intermediate precision (RSD %) were achieved by adding U-[$^{13}C_{20}$]-OTA (**Table 3**). Moreover, using an internal standard had the additional advantage that instrumental error during the analysis or human error during pretreatment could be detected by continuously checking the peak area of the internal standard when analyzing naturally contaminated samples.

The OTA contents of the naturally contaminated samples were compared between the methods with and without an internal standard. Assuming a 100% hypothetical recovery for the method using an internal standard, the average recovery for the method without an internal standard was 88% (range = 77−100%) and the regression curve showed a good correlation ($R^2$ = 0.984; **Figure 5**).

In summary, the analysis of both OTA-spiked samples and naturally contaminated samples showed better recoveries and precision when U-[$^{13}C_{20}$]-OTA was used as an internal standard.

**Table 4.** OTA Concentrations in Ready-To-Drink Coffees

| sample[a] | OTA (ng/mL) | sample | OTA (ng/mL) |
|---|---|---|---|
| 1 | 0.037 | 16 | 0.010 |
| 2 | 0.033 | 17 | 0.010 |
| 3 | 0.026 | 18 | trace[b] |
| 4 | 0.024 | 19 | trace |
| 5 | 0.024 | 20 | trace |
| 6 | 0.020 | 21 | trace |
| 7 | 0.018 | 22 | trace |
| 8 | 0.017 | 23 | trace |
| 9 | 0.017 | 24 | trace |
| 10 | 0.017 | 25 | trace |
| 11 | 0.016 | 26 | trace |
| 12 | 0.014 | 27 | trace |
| 13 | 0.014 | 28 | trace |
| 14 | 0.012 | 29 | trace |
| 15 | 0.012 | 30 | trace |

[a] Each sample corresponds to a different brand. [b] Trace values ranged from the LOD to 0.010 ng/mL (the lower limit of the calibration curve).

**Analysis of Commercially Available Samples.** The developed method was applied to 30 commercially available ready-to-drink coffees. The results of the analysis are summarized in **Table 4**. All of the analyzed coffee samples were found to be contaminated with OTA, and the highest concentration was 0.037 ng/mL, which corresponded to 7.1 ng/bottle. The intake of OTA from this product would therefore be no more than 0.8% of the current

provisional tolerable intake of 100 ng (kg bw)$^{-1}$ week$^{-1}$, even if an individual with a bw of 60 kg drank this beverage daily. This result suggests that the health risk to consumers from ready-to-drink coffees is relatively low. However, constant observation and further research are needed to estimate the total intake of OTA from food in Japan.

In summary, we developed an accurate and sensitive method, involving rapid and easy pretreatment, for the quantification of OTA. Using this approach, we found that the OTA intake from the ready-to-drink coffees analyzed in this study posed an insignificant health risk to consumers in Japan. Owing to its high selectivity, LC/MS/MS analysis could be a useful tool for analyzing mycotoxins, and we demonstrated the efficacy of this method for accurately evaluating the intake of OTA from coffee beverages.

## ABBREVIATIONS USED

U-[$^{13}C_{20}$]-OTA, uniformly stable isotope-labeled ochratoxin A; BW, body weight; CE, collision energy; CXP, collision cell-exit potential; DP, declustering potential; ESI, electrospray ionization; FIA, flow-injection methodology; HPLC, high-performance liquid chromatography; IARC, International Agency for Research on Cancer; JECFA, Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives; LC/MS/MS, liquid chromatography−tandem mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; MRM, multiple-reaction monitoring; MW, molecular weight; OTA, ochratoxin A; OTB, ochratoxin B; PBS, phosphate-buffered saline; PCB, polychlorinated biphenyl; PTFE, polytetrafluoroethylene; RSD, relative standard deviation.

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