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Development of a lyophilized soybean paste certified reference material for the analysis of ochratoxin A

Seonghee Ahn, Suyoung Lee[1], Sunyoung Lee, Byungjoo Kim*

Division of Metrology for Quality of Life, Korea Research Institute of Standards and Science, Yuseong, Daejeon 34113, Korea

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ABSTRACT

A certified reference material (CRM) [[2]KRISS CRM # 108-10-018] for the analysis of ochratoxin A (OTA) in doenjang (fermented soybean paste and popular food in Korea) was produced to ensure the reliability of analytical results in testing laboratories. A home-made doenjang was chosen as a raw material after testing its OTA level. The raw material was freeze-dried, pulverized, sieved and homogenized. An isotope-dilution-liquid chromatography/tandem mass spectrometric method (ID-LC/MS/MS) which was previously developed and validated in this laboratory was used as a higher-order reference method for characterization, homogeneity studies, and short-term stability studies. The CRM had good between-bottle homogeneity with 0.56% relative standard deviation among 10 selected units. The stability of the CRM at −70 °C (the storage condition in our laboratory) and at −20 °C (the possible storage temperature at user sites) were tested for up to 8 months. No change in the OTA content was observed within the measurement uncertainty. The stability of the CRM at room temperature (for regular use and transportation) was also tested and confirmed. The certified value was (49.50 ± 1.17) μg/kg, where the expanded uncertainty was in the confidence level of 95%.

1. Introduction

Ochratoxin A (OTA) is a mycotoxin produced by a variety of fungal species belonging to the genera *Aspergillus* and *Penicillium* (van der Merve et al., 1965; Geisen et al., 2004; el Khoury and Atoui, 2010) Such molds are virtually ubiquitous, and OTA frequently occurs in a large variety of foods, such as cereals, beans, spices, dried fruits, grapes and coffee when they are exposed to inappropriate conditions during storage and transportation. OTA is known to have nephrotoxic (Scott et al., 1998), teratogenic (Gilani et al., 1978), neurotoxic (Brown et al., 1976), genotoxic (Costa et al., 2016), carcinogenic (Bendele et al., 1985; Pfohl-Leszkowicz and Manderville, 2007) effects in humans and animals. A kidney disease referred to as Balkan endemic nephropathy is one of the well-known adverse human health effects of OTA. The tolerable daily intake of OTA has been set at 5 ng/kg bodyweight/day by the Scientific Committee for Food of the European Commission (Scientific Committee on Food Opinion on Ochratoxin A, 1998). To protect human health, thorough control of foods contaminated with OTA is essential and reliable analytical methods are required.

Doenjang is a traditional soybean paste fermented by ambient fungi in the genera *Aspergillus* and *Penicillium* is one of the most popular regional foods. OTA draws great attention from Korean regulatory bodies, since doenjang can be contaminated with OTA when it is not fermented under proper conditions. Doenjang is used alone or as a condiment or seasoning in a number of Korean foods. The maximum limit of OTA in doenjang and related foodstuffs is set at 20 μg/kg by Korean government. As doenjang is food with regional characteristics, our laboratory, the National Metrology Institute (NMI) of Korea, has to develop certified reference materials (CRMs) for the analysis of OTA in doenjang as CRM is a key tool to disseminate national measurement standards in chemical metrology area to ensure the reliability of testing laboratories for screening of OTA in doenjang products.

Various analytical methods are available for the analysis of OTA in foods and animal feeds. Enzyme-linked immunosorbent assay (ELISA) (Morgan et al., 1986; Zheng et al., 2005) has been used for rapid screening. Although it is easy to use, ELISA is vulnerable to matrix interference compared to other instrumental analytical methods. Thin layer chromatography (TLC) (Scott et al., 1970), high performance liquid chromatography (HPLC) (Call'Asta et al., 2004; Iqbal et al., 2016), and gas chromatography (GC) (Soleas et al.,

* Corresponding author.
E-mail address: Byungjoo@kriss.re.kr (B. Kim).
[1] Current address: Yuhan Corporation, Youngin, Gyeonggi-do, 446-902, Korea.
[2] KRISS:Korea research institute of standards and science.

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2001) with various detectors have been used to analyze OTA in foodstuffs. As LC/tandem MS instruments are getting widely available these days, LC/MS/MS methods became prior choices for the reliable determination of OTA in foodstuffs because they can provide high sensitivity and selectivity (Becker et al., 1998; Lau et al., 2000; Lindenmeier et al., 2004; Noba et al., 2009; Han et al., 2010; Saito et al., 2012;). Recently, our laboratory developed an isotope-dilution-liquid chromatography/tandem mass spectrometric (ID-LC/MS/MS) method as a higher-order reference method, which can provide measurement results without bias and with much smaller uncertainty than methods used by testing laboratories, for the accurate determination of OTA in doenjang as well as other foodstuffs (Ahn et al., 2016). The ID-LC/MS/MS method is to be used as a higher-order reference method for the value-assignment of OTA in food CRMs, which will help to ensure the reliability of measurement results of testing laboratories by using them as a quality control materials or calibration standards.

Two levels of wheat CRMs (Wood et al., 1997), wine and roasted coffee CRM (Koch et al., 2011) for the analysis of OTA were produced by Institute for Reference Materials and Measurements (IRMM). In addition to those food matrices, soybean-based fermented foods are popular in Asian countries and those foods are prone to be contaminated with OTA. Our laboratory launched a program to develop CRMs for the analysis of mycotoxins in food. The development of a doenjang CRM for the analysis of OTA was chosen as the first project due to its regional characteristic issues. We report the preparation and certification of a doenjang CRM for the analysis of OTA.

2. Materials and methods

2.1. Materials

OTA was purchased from Biopure (Tulln, Austria) and used as a primary reference material without further purification. The purity of the OTA was determined by the protocol developed and maintained in our laboratory. The purity of OTA was (98.5 ± 0.7) %. $^{13}C_{20}$-OTA was purchased from Biopure and used as the internal standard for the ID-LC/MS/MS analysis. HPLC-grade methanol and acetonitrile were obtained from Burdick and Jackson (Muskegon, MI, USA). Phosphate buffered saline (PBS) and formic acid were purchased from Sigma-Aldrich (St, Louis, MO, USA). OchraTest ™(1 cc) solid phase extraction (SPE) cartridges for sample clean-up were purchased from VICAM (Milford, MA, USA). A coffee CRM for Ochratoxin analysis was obtained from IRMM (Gill, Belgium) and used as a control material.

2.2. Preparation of candidate reference material

Several home-made doenjang products were obtained and tested as candidate raw materials for CRM production. All the doenjang products were soybean paste fermented by a traditional Korean method. The method of Korean style soybean paste is briefly described here as informations. Soybeans soaked with water are boiled and crushed as a paste form. Crushed bean paste is molded to cake form and fermented on the straw at dry and cold environment for several months. Fermented soy bean cakes was soaked in salted water and fermented furthermore for several months. After then, salted water (it is called soybean sauce) is removed and remaining soybean paste are aged for several months. Well aged soybean paste is called doenjang in Korea. Fermentation and aging conditions and times are varying depending on regions and homes. Based on preliminary test results, one doenjang product, which showed a naturally occurring OTA level of approximately 20 μg/kg was chosen as a candidate raw material. About 14 kg of the raw material was freeze-dried. The weight of the material was reduced to about 5.6 kg, 40% of its original weight. This dried doenjang was pulverized with a variable speed rotor mill (Pulverisette 14, Fritsch, Idar-Oberstein, Germany) and sieved using a vibrating sifter (V/SIFTER-141, Dae-ga Inc., Korea) to obtain doenjang powder with 50–250 μm particle size. Bulk doenjang powder was then homogenized with a 70 L V-blender (V/Mixer 70 L, Dae-ga Inc., Korea) for 15 h. The powder was bottled in clean amber bottles (Fisher, USA, wide mouth packers with PTFE faced PE-lined caps, 60) at around 20 g per unit by scooping appropriate amount from the bulk material and pouring into bottles. The bottles were purged with argon gas, and tightly sealed with Teflon-lined caps. A total of 270 units were prepared and stored at −70 °C in a deep-freezer. The material was assigned as KRISS CRM #108-10-018 (batch number 130312) “Fermented soybean paste (dried powder) CRM for the analysis of Ochratoxin A.”

2.3. Analytical method

The ID-LC/MS/MS method developed in our laboratory was used for characterization, homogeneity test, and stability test of the candidate doenjang CRM. Details for the ID-LC/MS/MS method are reported in another article (Ahn et al., 2016). A brief description and modification made for this study are described as follows. Four replicates of OTA standard solution (1 mg/kg) were gravimetrically prepared in a water:methanol (40:60, v/v) solution. Two isotope ratio standard (1:1) solutions from each of the four replicates were prepared by gravimetrically mixing with a $^{13}C_{20}$-OTA solution (70 μg/kg in water:methanol (40:60, v/v)). A total of eight isotope ratio standard solutions were cross-checked by the ID-LC/MS/MS method. Based on the cross-check results, one isotope ratio standard solution was chosen for the calibration of sample analysis. Full information for the validation of the method was described in the previous article (Ahn et al., 2016). The coffe CRM from IRMM was used as a secondary control material to check the performance the analytical method when it was required.

For sample analysis, 0.4 g of doenjang powder sample was placed in a 20 mL vial and spiked with an appropriate amount of the $^{13}C_{20}$-OTA solution to obtain an isotope ratio of 1:1. 8 mL of the acetonitrile:water extraction solvent (60:40, v/v) was added to the vial. The amount of the $^{13}C_{20}$-OTA solution to be spiked to sample was calculated based on preliminary test of OTA level in the sample by the same analytical methods. The vial was shaken for 15 min and centrifuged at 2500 × *g* for 5 min. The supernatant was collected in a new vial and evaporated under nitrogen at 45 °C for 30 min to reduce the volume of the extract to 1–2 mL. The extract was then diluted with 16 mL of PBS and filtered with Whatman GF/B glass microfiber. The filtrate was loaded on an OchraTest™ SPE cartridge, washed with 10 mL of water and then eluted with 3 mL of methanol. The eluent was dried under nitrogen (TurboVap® II, Biotage, Sweden) and reconstituted with 0.2 mL of water:methanol (40:60, v/v) containing 0.5% formic acid.

All analysis was carried out using a ThermoElectron TSQ Quantum mass spectrometer (San Jose, CA, USA) coupled with an electrospray ionization interface and a Waters ACQUITY UPLC system (Milford, MA, USA). A C18 LC Column (Waters, 3.5 μm, 2.1 × 100 mm) was used for LC analysis. Injection volume for sample extract and the calibration solution was 10 μL. The mobile phase consisted of solvent A (water with 0.5% formic acid) and solvent B (methanol). The mobile phase started with 60% B and a linear gradient was set from 60% B to 90% B from 0 to 12 min. The flow rate of the mobile phase was 0.25 mL/min. The mass spectrometer was operated in positive ion mode with selected reaction monitoring (SRM) of OTA at *m/z* 404 → *m/z* 239 and $^{13}C_{20}$-OTA at *m/z* 424 → *m/z* 250. The SRM channels of *m/z* 404 → 358 for OTA and *m/z* 424 →378 for $^{13}C_{20}$-OTA were used as confirmatory monitoring as describe in our previous article (Ahn et al., 2016).