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Effect of Pressure Cooking on Aflatoxin B_1 in Rice

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The effect of pressure cooking on aflatoxin residues in polished rice was conducted to determine reduction of aflatoxin and mutagenic potentials. Three rice lots consisting of naturally contaminated, *A. parasiticus*-infested, and aflatoxin-spiked rice were steamed by ordinary and pressure cookers after they were washed with water. They were chemically analyzed for aflatoxins using a silica solid phase extraction tube and high-performance liquid chromatography (HPLC)−fluorescence detection (FD), and the presence of aflatoxin residues was confirmed using HPLC−electrospray ionization (ESI)−mass spectrometry (MS). An in vitro mutagenicity test with *Salmonella typhimurium* TA100 was employed to verify the results based on chemical analyses. The aflatoxin loss (78−88%) was notable after pressure cooking, and the reduction of aflatoxin-induced mutagenic potential (68−78%) was in good agreement with the HPLC results. It can be concluded that Koreans are safe from the aflatoxin-related risk if a pressure cooker is employed for cooking rice. The average Korean daily intake of aflatoxin through the consumption of staple rice would fall to 0.15 ng/kg bw/day, which would not exceed the established tolerable daily intake (0.40 ng/kg bw/day).

KEYWORDS: Aflatoxins; pressure cooking; *Salmonella* mutagenicity

INTRODUCTION

The aflatoxins are a group of related mycotoxins produced mainly by *Aspergillus flavus* and *Aspergillus parasiticus* (*1*), ubiquitous storage fungi associated with peanuts, corn, and cereals (*2*, *3*); rice grains are occasionally contaminated (*4*−*6*). The consumption of corn highly contaminated with aflatoxins has been associated with an increased risk of human liver cancer and acute hepatitis in some regions of South Africa and China (*7*, *8*). Seventeen aflatoxins have been isolated, but only four, B_1, B_2, G_1, and G_2, are significant contaminants of food. Aflatoxin B_1 (**Figure 1**) is usually found in the greatest concentration in foods and is the most acutely toxic of the aflatoxins.

Rice has been the most important crop in the Korean food supply for thousands of years. Polished rice is sequentially subjected to rinsing with water and steaming prior to being consumed as cooked rice, the staple food in Korea. Our recent studies on dietary exposure of Koreans to aflatoxin B_1 from various commodities showed that even low concentrations aflatoxin B_1 (<10 ng/g) in rice could pose a potential health risk because of the large amount of rice consumed by Koreans (16% of the Korean total diet) (*5*, *9*). Furthermore, these reports also noted that rice is the major contributor of aflatoxin intake by Koreans.

Previously, there were few considerations of the effect of cooking on aflatoxins in rice, because they were assumed to be relatively heat stable once formed. A number of reports have revealed that thermal processes, i.e., frying, baking, or extrusion for making corn-based foods, could reduce concentrations of aflatoxins (*10*−*12*). In particular, the loss of aflatoxin B_1 during rice cooking was also reported as being between 6 and 88%, depending on the rice-to-water ratios used and whether the rice was cooked under pressure (*13*). However, these results were based only on conventional thin-layer chromatography (TLC) analysis. Using high-performance liquid chromatography with fluorescence detection (HPLC-FD) and HPLC coupled with mass spectrometry (HPLC-MS) as well as an in vitro *Salmonella* mutagenicity assay, we found that typical processes employed in Korea for making cooked rice (rinsing with water and steaming with an ordinary cooker) significantly reduced aflatoxin B_1 (mean reduction, 34%) in naturally contaminated polished rice and reduced mutagenicity by 27% (*14*). This suggested that the concentration of aflatoxin B_1 in cooked rice that reached the consumer was considerably lower than that in raw polished rice. Thus, the early estimate of Korean aflatoxin B_1 intakes from rice based on the concentration of aflatoxin B_1 in raw material such as polished rice was revised to reflect both chemical and toxicological variations of aflatoxin B_1 residues in cooked rice; the updated Korean daily intake of aflatoxin B_1 from the consumption of cooked rice ranged between 0.58 and

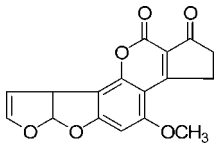

Figure 1. Structure of aflatoxin B_1.

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3.94 ng/kg body weight/day (*14*). Even though the Korean probable daily intake (PDI) of aflatoxin B_1 through rice consumption became lower than the previous estimate (*5*), the intake figures were still higher than the provisional maximum tolerable daily intake (TDI) of 0.40 ng/kg body weight/day for adults with hepatitis, indicating that Koreans are not secure against the threat of food-borne aflatoxin B_1 (*14*). The use of a pressure cooker instead of an ordinary cooker has been proposed to enhance the decomposition or removal of naturally incurred aflatoxins in cooked rice (*14*), as Rehana et al. (*13*) already found much more destruction of aflatoxin B_1 (>70%) by pressure cooking.

The objectives in the present study were to investigate the effect of pressure cooking on aflatoxin residues in three lots, which consisted of naturally contaminated, *A. parasiticus*-infested, and aflatoxin B_1 (50 ng/g)-spiked rice, as compared to that of ordinary cooking on the same lots by using both HPLC-FD and HPLC-MS, and to evaluate the processing toxicologically by using an in vitro *Salmonella* mutagenicity test (*15*), so that reduction of aflatoxin concentrations based on chemical analysis could be confirmed with bioassay findings.

MATERIALS AND METHODS

Safety. Aflatoxins are powerful hepatotoxins as well as human carcinogens and should be handled with care. All experiments were carried out in a fume hood.

Chemicals and Analytical Standard. HPLC grade solvents and analytical grade reagents were used for all purposes. Aflatoxin standards (aflatoxin B_1, B_2, G_1, and G_2) were all purchased from Sigma (St. Louis, MO), and a stock solution was prepared in acetonitrile/methanol (1:1), which was stored in an amber vial in a freezer (ca. −18 °C). Standard solutions for HPLC were prepared by appropriate dilution in the mobile phase, methanol/acetonitrile/water (17:17:70).

Sample Lots. A total of three lots (each 1 kg) of polished rice from our previous study were used in this experiment (*6*). All were harvested in 2002 in Korea and were obtained from several grain wholesale markets in Seoul. They were of acceptable visual quality and intended for human consumption. They consisted of a sample of rice naturally contaminated with aflatoxin B_1 at 6.7 ng/g; another aflatoxin-free rice lot (aflatoxin B_1 < 0.8 ng/g), which served as a control for recovery tests as well as a blank spiked with a known amount of aflatoxin B_1 at a final concentration of 50 ng/g; and an *A. parasiticus*-infested rice lot prepared as previously described (*6*). A 100 g amount of polished rice and 20 mL of distilled water were mixed in a 250 mL Erlenmyer flask. After the mixture was held at room temperature for 12 h, each flask was autoclaved for 30 min. Each flask was inoculated with a loop of *A. parasiticus* ATCC 15517 (ATCC, Manassas, VA) cultured on potato dextrose agar medium (Difco, Becton Dickinson, Sparks, MD) and incubated at 25 °C in the dark. For the first 7 days of the incubation period, each flask was shaken once a day. After 3 weeks, fungal-cultured rice was autoclaved intermittently twice a day and then used as an *A. parasiticus*infested rice lot for further cooking study. All lots did not contain other detectable mycotoxins, such as ochratoxin A, fumonisins, nivalenol, deoxynivalenol, and zearalenone, when measured by HPLC. They were stored in sealed plastic bags under refrigeration (4 °C). Recoveries of aflatoxins (aflatoxin B_1, B_2, G_1, and G_2) from the powdered samples of both ordinary-cooked and pressure-cooked aflatoxin-free rice lots were determined, based on triplicate analyses at a spiking concentration of 5 ng/g. After the spiked samples were left for 2 h in a fume hood, each was extracted, cleaned up, and subjected to HPLC.

Preparation of Cooked Rice. The cooking procedure was performed according to the method of Park et al. (*14*). A 100 g amount of each rice lot was rinsed three times with 200 mL of distilled water and drained on a screen. Washed rice grains were combined with 200 mL of distilled water. Each mixture was subjected to different types of cooking; one (ordinary cooking) was cooked at 160 °C for 20 min in a commercial electric cooker (Samsung Electronics, CheonAn, Korea), and the other (pressure cooking) was cooked under the same conditions in a commercial electric pressure cooker (Samsung Electronics) fixed at 15 lb/in^2 (0.10 MPa). After cooling, the cooked rice was frozen overnight at −70 °C. All of the samples were freeze-dried with a laboratory lyophilizer (Millrock Technology Inc., Kingston, NY) and ground to a fine powder using a mortar and pestle. The powdered samples kept in a refrigerator (4 °C) were weighed and then analyzed for aflatoxin B_1 residues. The following experiments were done in two series of triplicates.

Aflatoxin Determination. The method employed was based on reversed phase HPLC-FD and solid phase extraction (SPE) cleanup as previously described (*6*, *14*). Five grams of the sample was extracted with 25 mL of methanol/water (8:2) by shaking for 30 min in an orbital shaker and then filtered through #4 paper (Whatman, Clifton, NJ). The remaining solid was extracted again and then also filtered. The first and second extracts were combined and partitioned with dichloromethane followed by a cleanup using Sep-Pak silica SPE tubes (Waters, Milford, MA) (*14*). These purified extracts were analyzed by HPLC at a flow rate of 1.0 mL/min. Precolumn derivatization was performed with trifluoroacetic acid using FD with a model 474 detector (Waters) with excitation set at 360 nm and emission set at 440 nm. Separation was done on a 150 mm × 3.9 mm i.d., 5 μm, Nova-Pak C_{18} column (Waters), and the mobile phase was methanol/acetonitrile/water (17:17:70).

To confirm the aflatoxin B_1 residues in the samples, HPLC-MS analysis was performed by a minor modification of a method described by Park et al. (*14*) with a VG Biotech platform MS (VG Biotech, Cheshire, United Kingdom) with electrospray ionization in positive ion mode; the cone voltage (90 eV) and the source temperature (120 °C) were applied, and the flow rates of drying gas and nebulizing gas (N_2) were 7.0 and 2.5 L/min, respectively. Each cleaned-up extract that contained aflatoxin B_1, as determined by HPLC-FD, was re-evaporated and then dissolved in an appropriate mobile phase of acetonitrile/methanol/10 mM ammonium acetate (2:6:15). The MS platform interfaced with an HP 1100 LC system (Agilent Technologies, Palo Alto, CA), which was equipped with a 150 mm × 2.0 mm i.d., 5 μm, UltraCarb ODS 30 column (Phenomenex, Torrance, CA), pumped at a constant flow rate of 0.2 mL/min. Mass spectra were obtained by scanning from *m*/*z* 250 to 750. Base peak ions of aflatoxin B_1 (*m*/*z* 313), B_2 (*m*/*z* 315), G_1 (*m*/*z* 329), and G_2 (*m*/*z* 331) were monitored in selected ion-monitoring mode. The detection limit obtained at a signal-to-noise ratio of 5:1 for aflatoxin B_1 by HPLC-FD was 0.8 ng/g and that with HPLC-MS was 0.1 ng/g.

Mutagenicity Assay. The Ames test was performed with *Salmonella typhimurium* TA100 according to the method of Park et al. (*14*). The effect of cooking on the mutagenicity of aflatoxin B_1 residues in three different rice lots was determined using a preincubation procedure in the presence of a rat liver S9 mix (XenoTech LLC, Lenexa, KS) as an external enzymatic metabolizing system. The methanol/water extracts obtained from powdered samples in each lot were evaporated to dryness under nitrogen gas, and the residues were reconstituted in 1 mL of dimethyl sulfoxide (DMSO). An extract from the aflatoxin-free rice lot served as a solvent control and was evaporated and redissolved in DMSO. These extracts were preincubated at 37 °C for 30 min with S9 mix and the bacteria (about 10^8 CFU/mL). After the preincubation period, the mixtures were diluted with soft agar and subsequently plated onto minimal glucose agar (Difco) plates. The number of histidine-positive revertants was counted after 2 days of incubation at 37 °C. A doubling of the number of spontaneous revertants was considered a positive mutagenic response using this bioassay.

Statistical Analyses. Statistical analyses were performed with Sigma Stat (version 3.0, Jandel Scientific, San Rafael, CA). The aflatoxin B_1 concentrations and the number of revertants were evaluated using a one-way analysis of variance. Differences among sample groups were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Recoveries of Aflatoxins from Cooked Rice. The overall mean recoveries of aflatoxin B_1, B_2, G_1, and G_2 from artificially spiked aflatoxin-free rice samples, which were powdered after

Table 1. Aflatoxin B_1 Residues in Samples of Three Polished Rice Lots before and after Cooking with Ordinary and Pressure Cookers, as Determined by HPLC-FD and HPLC-MS[a]

	aflatoxin B_1 (μg)	reduction (%)	
	raw rice	ordinary-cooked rice	pressure-cooked rice
lot 1[b]	0.54 ± 0.04	33	78[c]
lot 2[b]	4.20 ± 0.18	36	88[c]
lot 3[b,d]	813 ± 17.30	31	83[c]

[a] All of the values are corrected for recovery. The amount of aflatoxin B_1 was determined by multiplying AFB_1 concentration (ng or μg/g) by the total dry weight (g) of each sample from each lot ($n = 6$). [b] Lota 1, 2, and 3 are naturally contaminated, aflatoxin B_1 spiked, and *A. parasiticus* infested rice, respectively. [c] $P < 0.05$, significantly different from the amount present in both the raw- and the ordinary-cooked rice samples of the respective rice lots. [d] This lot contains some other detectable aflatoxins (214 ± 8.31 μg of aflatoxin B_2 and a trace amount of aflatoxin G_1).

Table 2. Aflatoxin B_1-Induced Mutagenic Potential in Samples of Three Polished Rice Lots before and after Cooking with Ordinary and Pressure Cookers, as Determined by the Ames *Salmonella* Test[a]

	reduction of aflatoxin B_1-induced mutagenic potential (%)	
	ordinary-cooked rice	pressure-cooked rice
lot 1[b]	23	71[c]
lot 2[b]	29	78[c]
lot 3[b,d]	19	68[c]

[a] All of the values are means obtained from two series of triplicate in each lot ($n = 6$). [b] Lots 1, 2, and 3 are naturally contaminated, aflatoxin B_1 spiked, and *A. parasiticus* infested rice, respectively. [c] $P < 0.05$, significantly different from the amount present in the ordinary-cooked rice sample of the respective rice lots. [d] This lot contains some other aflatoxins (214 ± 8.31 μg of aflatoxin B_2 and a trace amount of aflatoxin G_1).

each cooking procedure, varied from 81 to 88% by HPLC (data not shown). Sep-Pak silica SPE tubes (Waters) performed well for the analysis of cooked rice, all giving good recoveries (>80%) of added aflatoxins with low relative standard deviations for within-day repeatability (RSD) ranging from 3 to 6%. These results are in agreement with previous work (*14*).

Aflatoxin B_1-Induced Mutagenicity. The Ames test to *S. typhimurium* TA100 was performed. Aflatoxin B_1 had a mutagenic effect on *S. typhimurium* TA100 when mixed with the S9 enzymes, and there was a significant concentration dependence ($R^2 = 0.90$) of the mutagenic potential in the range of 20−50 ng/plate (data not presented). Moreover, it was proved that the concentrated cooked rice extracts (in DMSO) prepared from aflatoxin B_1-free rice lots, which served as the matrix and solvent control, appeared to have a negative mutagenic response. These findings are also consistent with our recent study (*14*).

Effect of Ordinary- and Pressure-Cooking on Aflatoxin B_1 in Rice. The cooking process usually used in Korea for cooking rice, which involves water washing and steaming at 160 °C, showed significantly reduced aflatoxin residues in the final cooked rice (31−36% loss) as measured by HPLC-FD (**Table 1**). Initial aflatoxin contents in the polished rice appeared to have no discriminating effects on the results when the three lots were compared. In the case of lot 3 prepared from *A. parasiticus*-infested rice, other aflatoxins such as aflatoxin B_2 and G_1 were detected by HPLC-FD followed by MS; the ratios of each aflatoxin B_2 and G_1 to B_1 were about 1:4 and 1:100, respectively; also, the percentages of aflatoxin B_2 and G_1 reduction after cooking with both cookers were parallel with that of aflatoxin B_1 (data not shown). The loss of aflatoxin was considerably higher ($P < 0.05$) when using a commercial pressure cooker (approximately 15 lb/in^2) at 160 °C for 20 min (78−88% loss) than the ordinary cooker without pressure under similar conditions (31−36% loss). The effect of steaming at 160 °C by ordinary cooker on aflatoxin residues in the cooked rice was already reported by others including us (*14*, *16*). The appreciable effect on aflatoxin of the pressure cooking is in line with the work accomplished by Rehana et al. (*13*), who investigated a pressure-cooking process on water-washed white rice that contained or was spiked with aflatoxins: They showed that pressure cooking left only 12% of the aflatoxins in the cooked rice. Another study done by L'vova and co-workers (*16*) is more clear-cut with regard to increased loss of aflatoxin B_1 with pressure cooking, namely, 56% as compared to 37% with ordinary cooking. However, reliable data on the residual levels of aflatoxin in pressure-cooked rice have not yet been available because a conventional TLC was used in the above publications as the only analytical method and no confirmation by any other chemical analyses such as HPLC-FD or MS was made. Among several heat process operations, extrusion cooking could correspond to the pressure cooker, because it also generates high temperature and pressure. There have been two studies dealing with the effects of extrusion on reduction of aflatoxins recently published (*11*, *12*). Extrusion of the raw corn flour artificially spiked with aflatoxin B_1 at 50 ng/g reduced the level only by 10%, whereas significant losses (46%) were observed when corn flour naturally contaminated with aflatoxin B_1 at 0.49 μg/g was extruded without any addition of calcium hydroxide or hydrogen peroxide.

Loss of aflatoxins during processing might occur because it is washed away, destroyed, bound to a food matrix, or changed to unknown decomposed products. In the latter two cases, it cannot be assumed that reduction in aflatoxin levels means decreased toxicity. For example, the presence of bound fumonisin (a *Fusarium* mycotoxin) conjugated with corn components in heat-processed corn-based foods was reported recently (*17*). In treatment with ammonia for detoxifying aflatoxin-contaminated corn to be used for animal feeds, Park et al. (*18*) identified breakdown products of aflatoxins and also evaluated lower levels of aflatoxin B_1 by an animal feeding study.

Few studies so far have looked at the toxicological fate of aflatoxin during food processing. The mutagenic potential of aflatoxins using the Ames test should be carried out not only to clarify if a remarkably reduced aflatoxin residue in pressure-cooked rice also decreases the aflatoxin-induced mutagenicity to *S. typhimurium* TA 100 but also to estimate possible interactions in the biological system, although there were no decomposition products found in the mass range of the HPLC-MS analysis. When comparing the mutagenic potential of cooked rice samples in three rice lots (naturally contaminated, *A. parasiticus*-infested, and aflatoxin B_1-spiked rice), it was found that the greater the percentage of loss of aflatoxins, the lower the *Salmonella* mutagenicity is (**Table 2**), which is consistent with the previous publication; 27% reduction of mutagenic potential of the ordinary cooked lots was already reported (*14*). The percentage of mutagenicity of both cooked rice samples seemed to be lower than those of their corresponding raw samples in the respective rice lots; however, the difference was significant ($P < 0.05$) only for the pressure-cooked rice. Lot 3 showed low reduction of mutagenic potential as compared with the others, even though there was no notable difference found between values. It appears to be due to other aflatoxins such as aflatoxin B_2 and G_1 remaining even after the

Table 3. Revision of the Korean Dietary Intake of Aflatoxin B_1 through Staple Rice Consumption, Based on the Percentages of Aflatoxin Reduction Determined Chemically and Toxicologically in Polished Rice during Steaming with a Pressure Cooker

	mean aflatoxin B_1 (ng/g)[a]	aflatoxin B_1 intake (ng/kg bw/day)
polished rice[b]	0.20	0.89
ordinary-cooked rice[b]	0.13	0.58
pressure-cooked rice[c]	0.03	0.15

[a] In determining the mean, samples below the level of detection were taken as zero. [b] Values in our previous publication (*14*). [c] Mean reduction of aflatoxin B_1 during pressure cooking of polished rice ranges from 72 to 83%, respectively, as determined by chemical and toxicological analyses.

cooking process; they can act as mutagens in the Ames test, despite a lower potency than aflatoxin B_1 (*19*). These findings indicated that the pressure-cooking procedure evaluated in this study greatly diminished aflatoxin-induced toxicity as compared with ordinary cooking. Accordingly, it is likely that the analytical results were consistent with the in vitro biological findings. If polished rice is cooked under pressure, therefore, a much bigger reduction in aflatoxin can be expected.

Revision of the Korean Dietary Intake of Aflatoxins through Pressure-Cooked Rice Consumption. The above results provoked reassessment of the Korean daily intake of aflatoxin through consumption of staple rice estimated in our previous reports (*14*). The mean aflatoxin B_1 content had been calculated by assuming that the level of aflatoxin B_1 in samples below the detection limit was equal to zero. **Table 3** illustrates that dietary intakes of aflatoxin from the consumption of rice by the average Korean dramatically diminished when the percentage of aflatoxin reduction in pressure-cooked rice was applied to the aflatoxin concentrations in polished rice based on survey data. The Korean PDI of aflatoxin B_1 through consumption of pressure-cooked rice was 0.15 ng/kg bw/day; this is lower than the previous estimate based on the percentage of aflatoxin reduction during steaming with an ordinary cooker and would be much lower than the provisional maximum TDI of 0.40 ng/kg of body weight per day for adults with hepatitis (*20*), indicating that Koreans could be safe from aflatoxin-related risk if a pressure cooker were to be generally employed for cooking rice. It has been estimated that the mean dietary intakes of total aflatoxin are 0.15 ng/kg bw/day for Australians, 0.80 ng/kg bw/day for Swedes, and 0.26 ng/kg bw/day for Americans, respectively (*21*, *22*). In comparison with other countries, intake of aflatoxin B_1 in Korea seems to be comparable with those in certain other European and North American areas, even assuming that staple rice is a sole contributor to the intake of Koreans. In general, dietary exposure to mycotoxins occurs during the lifespan of humans. Therefore, if mean consumption data as well as mean body weight of Koreans in the same age bracket are available, the exposure of Koreans to aflatoxins within a lifespan could be plotted as a function of age. Fitting these data to a regression curve ($r^2 = 0.79$) yields the results presented in **Figure 2**, showing that the relative intake tends to decrease with age. Toddlers (1−2 year old children) appear to be the most susceptible group in Korea, with other age groups consuming less than this; the highest consumption of polished rice relative to body weight is noted in this group. Korean exposure to dietary aflatoxin B_1 would be unlikely to reach the provisional TDI (0.40 ng/kg bw/day). However, for the most vulnerable group in Korea, the margin between the PDI of aflatoxin B_1 and the provisional TDI would be rather small, especially for toddlers with a high consumption of staple rice, indicating that further discussion for a more restricted action level for aflatoxin in polished rice is needed.

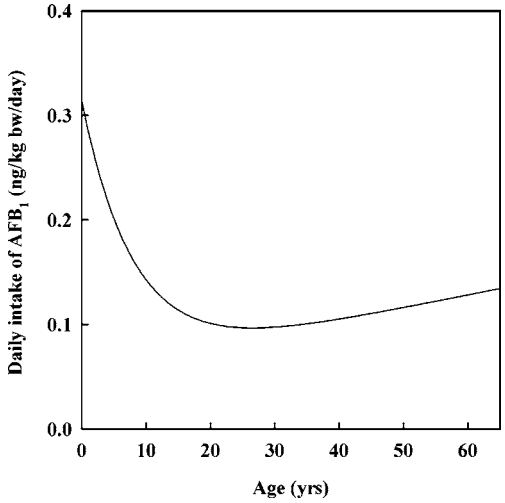

Figure 2. Distribution of Korean daily intakes of aflatoxin B_1 from the consumption of staple rice as a function of age, assuming it is cooked with a pressure cooker.

The present study gives us considerable information on the fate of aflatoxin B_1 during the pressure cooking of rice. Conditions typically used in Korea for cooking rice with a pressure cooker caused loss of aflatoxin B_1 (72−83%) as determined chemically and toxicologically, probably by extraction into the drained water or by decomposition of the toxins. These results provide evidence for sufficient potential removal of aflatoxin B_1 causing health risks to Koreans that depend on rice as a dietary staple. To date, steaming with a pressure cooker appears to be a promising method of removing aflatoxin in raw rice, and this processing makes cooked rice without a loss of flavor and nutrients. Furthermore, as reviewed recently by Castells et al. (*23*), a heat process such as extrusion cooking corresponding to the pressure cooker can be applied widely for industrial food processing in order to reduce the health risk to consumers of aflatoxins remaining in foods. This report shows the estimate of Korean PDI only based on the consumption of rice. Therefore, more studies are needed to determine both the aflatoxin B_1 contamination levels and the processing effects on aflatoxin B_1 residues in various foodstuffs, which contribute to the Korean PDI of aflatoxin.

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