

コメおよびソバ中のオクラトキシン分析法の単一試験所による妥当性確認

誌名	マイコトキシン
ISSN	02851466
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巻/号	57巻2号
掲載ページ	p. 81-86
発行年月	2007年7月

Single laboratory validation for a method of analysis for ochratoxin A in rice and buckwheat

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Abstract

Ochratoxin A (OA) is a mycotoxin produced by certain *Aspergillus* and *Penicillium* fungi. Contamination with OA can occur in many foods such as cereals, coffee, wine, dried fruits, and meat products. However, little is known about ochratoxin contamination on rice, which is a staple food for many Asian countries. In this study we examined a method of analysis for OA in rice and buckwheat. Five types of samples, polished japonica type rice (short grain), polished glutinous rice, rough japonica type rice, rough indica type rice (long grain) and buckwheat (two samples) were tested. Samples were spiked with OA over the range from 0.1 to 10.0 µg/kg for the linearity study and at 0.5 and 5.0 µg/kg for the reproducibility study. Ochratoxin A was extracted with acetonitrile/water, isolated using an immunoaffinity column, separated by HPLC, and detected by fluorescence. At 0.5 µg/kg level, within day recoveries were 64.5 to 110.2 % and Relative Standard Deviations (RSDs) were 5.7 to 16.7 % and between days, recoveries were 55.8 to 99.6 % and RSDs were 4.3 to 15.0 %. Average recoveries of OA in the range of 0.2 to 10 µg/kg were 89.6 to 118.1 %. From these result, it is shown that this method can be applied for the analysis of OA present as a contaminant in rice and buckwheat in the range from 0.2 to 10 µg/kg.

Key words : ochratoxin A, immunoaffinity cleanup, HPLC, fluorescence, validation

(Received: January 25, 2007, Accepted: May 1, 2007)

Introduction

Ochratoxin A (OA) is a mycotoxin which is produced by some *Aspergillus* and *Penicillium* fungi. Ochratoxin A is nephrotoxic as well as carcinogenic¹⁾, and is often found in cereals, coffee, wine and animal products^{2,3)}. Thus, the prevention of OA contamination of agricultural products is an important health concern⁴⁾. Because of its widespread distribution and toxicity, the regulation in EU for OA contamination of foodstuffs has become very strict⁵⁾, requiring sensitive and accurate analytical methods. Current methods for ochratoxins use either an immunoaffinity column⁶⁾ or a multifunctional clean up column⁷⁾ as a clean up process before the definitive HPLC analysis of ochratoxins.

However, only limited information for OA contamination on rice, a staple food for most Asian countries, is available because of the lack of validation of methods in this food crop. To validate a method, a full collaborative validation process such as the AOAC-OMA⁸⁾ is well known, although this

process is time and cost consuming. Given these limitations, in this study, we completed a single laboratory validation (SLV)^{9,10} of the analysis method of OA in rice and buckwheat as a matrix extension with minor modifications of the existing methods.

Materials and Methods

Sample For SLV study, two short grain rice, one is polished and the other is rough, one polished sweet rice, one rough long grain rice and two buckwheat samples were used. Rough rice samples were donated from Dr. Otsubo, National Food Research Institute, Japan. Ochratoxin A was spiked to rice or buckwheat samples about 16 hours prior to analysis. Two rice and buckwheat samples used for natural contamination study were purchased from local markets (Nagano, Japan) in 2004 and 2005 and also some rice samples were purchased at Bangkok, Thailand in December 2005. Samples were ground and stored at -20 °C until analyzed.

Chemicals Ochratoxin A was purchase from Sigma Chemical Co. Ltd (MI, USA). For OA stock solution, 5.0 mg of OA was dissolved in 5.0 ml of toluene/acetic acid (99:1, v/v). An aliquot of OA stock solution was placed into a glass vial and dried under N₂ gas stream. The working solutions for both analysis standards and spike solutions were prepared as acetonitrile solutions. For cleanup purposes OchraTest (VICAM, MA, USA) immunoaffinity column (IAC) and MycoSep #229 (Romer Labs., MI USA) multifunction clean up column (MFC) were used. All other chemicals used were either HPLC or GR grade.

Extraction and Clean up A twenty-five gram portion of the rice or buckwheat sample was measured into a 300 mL Erlenmeyer flask, 100 mL extraction solvent (acetonitrile/water, 6:4 (v/v)) was added, and the mixture shaken for 15 min by a shaker (model SA-31 Yamato, Tokyo, Japan). The extract was filtered through filter paper (Whatman No. 113) and 4 mL of filtrate was placed into a 100 mL glass flask. The extract was mixed with 44 mL phosphate buffered saline (PBS) and the complete PBS diluted extract was passed through an IAC. The IAC was washed with 10 mL water and the OA was eluted with 4 mL (1 mL × 4) methanol. The methanol was evaporated under N₂ gas stream, and the residues were dissolved in 1 mL of injection solvent (methanol/water/acetic acid. 30/70/1, v/v/v).

HPLC analysis Ochratoxin A was analyzed by HPLC (Shimadzu LC-6A series, Shimadzu, Kyoto, Japan) with fluorescence detector (RF-550, Shimadzu) both with and without post column alkaline treatment. The mobile phase contained acetonitrile, water and acetic acid (500:500:1, v/v/v). A 200 µL aliquot of extract was injected into the HPLC and ochratoxin A was separated by an ODS column (Inertsil ODS-2, 4.6 mm i.d. x 250 mm, GL Science, Tokyo, Japan). The flow rate of the mobile phase was 1.0 mL/min and alkaline solution (NaOH, 0.1 mol/L) was added at 0.3 mL/min using a NP-KX-110U Mini-Chemi Pump (Nippon Seimitsu Kagaku, Tokyo, Japan) as the post column treatment. Ochratoxin A was determined by fluorescence detector with excitation/emission wavelength 385/444 or 336/464 nm with or without alkaline solution respectively.

Results and discussion

MFC clean up A MFC column was tested to clean up rice samples following analysis conditions

as instructed by the column manufacture, and analyzed by HPLC. Using this clean up method developed for barley samples, OA can be successively analyzed within 15 minutes. However when this method was applied to rice samples, about two hours after the OA peak was detected, a large unknown peak appeared on the chromatogram. This peak made it more difficult to analyze OA in rice efficiently. Therefore we concluded that this clean-up method is not practical as a routine method for analysis of rice samples.

Effect of alkaline treatment Addition of alkaline solution enhanced OA peak height and area about 40 % and also decreased background noise on the chromatogram. This enhanced analysis helped to lower the detection and quantitation limit of OA. Also, use of the alkaline solution is effective for chemical confirmation of OA.

Linearity of standard curve Ochratoxin A standard solution was analyzed by HPLC. As shown in Fig. 1, the standard curve for OA is linear from 0.01 to 10.0 ng per injection, equivalent to 0.05 to 50.0 $\mu\text{g}/\text{kg}$ of OA in sample.

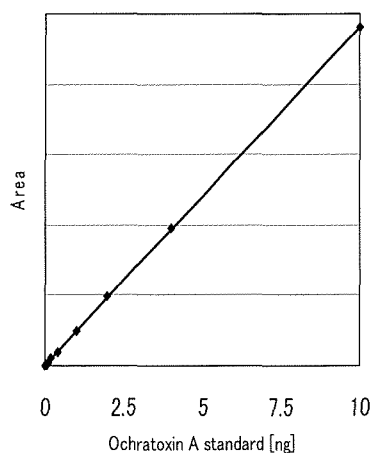


Fig. 1. Standard curve of ochratoxin A

Within day and day-by-day recovery test Rice and buckwheat samples were spiked with the standard OA solutions at 0.5 and 5.0 $\mu\text{g}/\text{kg}$ level, kept at 5 °C in the dark overnight (ca 16 hours), and analyzed by IAC clean up and HPLC detection methods. Six samples for each matrix were analyzed at the same time and designated as the 'within day study', and the same matrix samples were analyzed six times on different days for the 'day-by-day study'. As shown in Table 1, average recovery and relative standard deviation (RSD) of OA for 'within days' samples spiked at 0.5 or 5.0 $\mu\text{g}/\text{kg}$ were 87.1 % and 11.1 % or 82.8 % and 7.7 % respectively, and for 'day-by-day' samples, they were 84.0 % and 9.6 % or 89.6 % and 8.5 % respectively.

During this study, recoveries of OA from buckwheat samples were low compared to the rice samples. In evaluating the relation between recovery and the time after spiking OA to the sample, a decrease of recovery with increasing time after being spiked was observed (Table 2). In this study, recovery of OA from the buckwheat sample spiked about 16 or 40 hours before analysis were around 60 %, and recovery of OA from a buckwheat sample spiked within 20 min were about 90 %. From

Table 1. Comparison of recovery of ochratoxin A

Samples	Within Day (N=6)				Day-by-Day (N=6)			
	0.5 µg/kg		5.0 µg/kg		0.5 µg/kg		5.0 µg/kg	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Polished Rice (short)	90.0	5.7	84.7	6.4	99.6	6.2	92.3	4.0
Polished sweet rice	82.6	9.2	75.4	8.6	82.4	4.3	92.7	5.2
Rough rice (short)	96.6	10.7	94.0	3.8	94.5	9.7	102.9	3.2
Rough rice (long)	110.2	16.7	94.0	3.7	94.8	11.9	101.1	3.6
Buckwheat (1)	64.5	12.5	69.0	11.6	55.8	10.2	67.9	20.2
Buckwheat (2)	78.7	11.9	79.9	11.9	77.1	15.0	80.9	10.4
Average	87.1	11.1	82.8	7.7	84.0	9.6	89.6	8.5

Table 2. Effect of time after spike ochratoxin A to sample

Sample*1	Recovery (%)
40 hours (1)	66
40 hours (2)	64
16 hours (1)	74
16 hours (2)	52
20 min (1)	87
20 min (2)	91

* Buckwheat sample was spiked with OA at 0.5 µg/kg and analyzed after time shown as sample

these result, the low recovery of OA from buckwheat was considered due to the nature of the sample rather than the analytical method. Also, for a buckwheat sample (buckwheat (2) in Table 1) that was spiked 16 hours prior to analysis, the average recovery and RSD were considered acceptable.

Test for recovery at different concentrations Six different concentration of OA (0.1 to 10.0 µg/kg) was spiked to six samples (four rice samples and two buckwheat samples) and the recovery measured. Each matrix had three levels of OA and each level of OA was spiked for three matrices. The average recovery of 0.1 µg/kg concentration sample was over 200 % but the other five concentrations (0.2 to 10.0 µg/kg) were between ca 90 to 118 % (Table 3). Recovery of a rough rice sample at 0.2 µg/kg showed high recovery (ca 170 %). From results from other rough rice studies, two possibilities were suggested for the reasons of this recovery. One is low level of natural contamination and the other is background noise from the chromatogram. However, even including this data, the average recovery level is acceptable for this single laboratory validation (SLV) purpose.

Analysis of commercial rice and buckwheat samples Eleven rice samples and eight buckwheat samples were analyzed using the method described above. No OA was found in rice samples analyzed (Fig. 2A) but low levels of OA were detected from four buckwheat samples in the range of 0.2 to 2.4 µg/kg (Fig. 2B). Although it was not determined whether these buckwheat samples were produced domestically, this data suggest the need for monitoring OA contamination in buckwheat.

Table 3. Recovery of ochratoxin A at different concentrations

	0.1 µg/kg	0.2 µg/kg	0.5 µg/kg	1.0 µg/kg	5.0 µg/kg	10.0 µg/kg
Polished Rice(short)		108.5		90.3		91.2
Polished sweet Rice	108.7		101		90.3	
Rough rice(short)	197.5		94.7		97.9	
Rough rice (long)		172.7		105.9		104.0
Buckwheat(1)		73.1		82.1		91.7
Buckwheat(2)	327.8		74.2		80.5	
Average	211.3	118.1	90.0	92.8	89.6	95.7

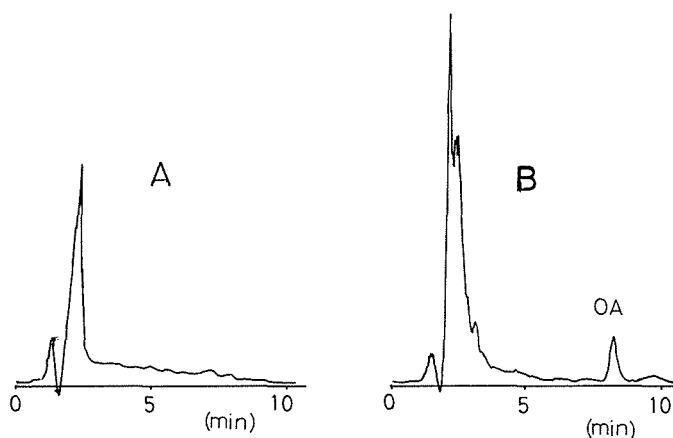


Fig. 2. Chromatogram of rice and buckwheat extract

A: Unpolished Japonica rice

B: OA naturally contaminated (2.4 µg/kg) buckwheat

Conclusion

This SLV study showed that ochratoxin A in rice and buckwheat was successfully analyzed by acetonitrile/water extraction with IAC clean up followed by HPLC with fluorescence detector in the range of 0.2 to 10.0 µg/kg. Rice and buckwheat samples purchased in local markets were analyzed and no detectable level of OA was found in rice samples but low levels of OA were found in some buckwheat samples.

This study was funded by the National Food Research Institute, Japan as a part of Research project "Integrated Research Program for Functionality and Safety of Food toward an Establishment of Healthy Diet". Authors thank Dr. Mark Fukayama for his editorial efforts on this manuscript.

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コメおよびソバ中のオクラトキシン分析法の単一試験所による妥当性確認

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オクラトキシン A (OA) はアスペルギルス属とペニシリウム属の一部の菌が作るマイコトキシンで、穀類、コーヒー、ワイン、乾燥果実や肉製品など多くの食品を汚染する。しかしアジアの人にとっての主食である米のオクラトキシン汚染はあまりわかっていない。ここでは、ジャポニカタイプの白米と玄米、餅米、インディカタイプの玄米とソバの5種類の試料を用いて、コメとソバ中のオクラトキシン A の分析法を検討した。試料に 0.1 - 10.0 µg/kg の OA を添加しての直線性試験と、0.5 と 5.0 µg/kg の OA を添加しての日内と日間の再現性試験を行った。OA はアセトニトリル / 水で抽出、イムノアフィニティークラムで精製後、蛍光検出 HPLC で分析をした。0.5 µg/kg 濃度での回収率とその相対標準偏差は、日内が 64.5 - 110.2 % で 5.7 - 16.7 %, 日間が 55.8 - 99.6 % で 4.3 - 15.0 % であった。0.2 - 10.0 µg/kg の範囲での回収率の平均は 89.6 - 118.1 % の範囲であった。これらの結果から、この方法によって、0.2 - 10.0 µg/kg の範囲でコメ (白米, 玄米) とソバ中のオクラトキシン A を分析できることが確認された。

キーワード: オクラトキシン A, イムノアフィニティークリーンアップカラム, HPLC, 蛍光, 分析法妥当性確認