International Journal of ChemTech Research
CODEN (USA): IJCRGG, ISSN: 0974-4290, ISSN(Online):2455-9555
Vol.9, No.11 pp 164-170, 2016

# Screening of ochratoxin A and B contaminated in dried chili using HPLC-fluorescence and liquid-liquid extraction

**Nalinthip Rotsisen[1], Chaturong Kanchai[1], Thanapat Sastraruji[2] and Churdsak Jaikang[1]***

[1]Department of Forensic medicine, Faculty of Medicine, Chiang Mai University, Chiang Mai, 50200, Thailand
[2]Dentistry Research Center, Faculty of Dentistry, Chiang Mai University, Chiang Mai 50200 Thailand
[1]Toxicology Section, Department of Forensic Medicine, Faculty of Medicine, Chiang Mai University, Chiang Mai, 50200, Thailand

**Abstract :** The aim of this study was validate method and simultaneously screen mycotoxins, ochratoxin A (OTA) and ochratoxin B (OTB), in dried chili and dried chili powders using High performance liquid chromatography with fluorescence detection (HPLC–FLD) and liquid-liquid extraction.Diflunisal was used as internal standard for validation method. Linearity, average recovery, limit of quantitation (LOQ), limit of detection (LOD) and precision were established validation parameters. The results showed that linearity in range 0.5- 100 ng/kg has $R^2$ more than 0.9950, average recovery was 87.06 – 94.86%, LOQ was 0.5 and 0.75 ng/kg and LOD was 0.25 and 0.50 ng/ kg for OTA and OTB, respectively while precision was shown asHorrat's ratio with the value less than 2. Sixty eight samples of dried chilies and dried chili powder were bought in local Chiang Mai markets during March and April 2016. The samples were extracted 3 times using ethyl acetate and the extract was screened for the OTA and OTB levels. It found that only 5 samples were contaminated both the OTA and the OTB but the levels were lower than permissible limits established by European Unit (EU), indicating that they were safety for consumers. The others samples, five dried chili powers were contaminated with the OTA in higher level than the permissible limits established by EU. This validated method is suitable for quantifying the OTA and OTB. However, the positive screening should be confirmed with solid phase extraction or another HPLC condition for confirming the OTA and OTB levels.
**Keywords :** OchratoxinA,Ochratoxin B, Chili, HPLC-FLD.

## Introduction

Ochratoxins are type of primarymycotoxin that produced by *Aspergillus ochraceus*, mainly in tropical and warmer regions and by *Penicillium verrucosum*, in colder areas[1] .The ochratoxins have many isoforms including ochratoxinA, ochratoxin B, ochratoxin C, ochratoxin α and ester forms[2-4] and the structures are shown in Fig.1.

Both ochratoxin A (OTA) and ochratoxin B (OTB) have been concerned because OTA is related to both urothelial urinary tract tumors and Balkan endemic nephropathy and the more frequent metabolite present in contaminated in foods [5-8]. OTB is caused of nephrotoxicity and renal toxicity[9]. The International Agency for

Research on Cancer classified OTA as a possible group 2B human carcinogen[10]. The Scientific Committee for Food of the European Commission has established a tolerable daily intake of OTA at 5 ng/kg body weight/day[11].

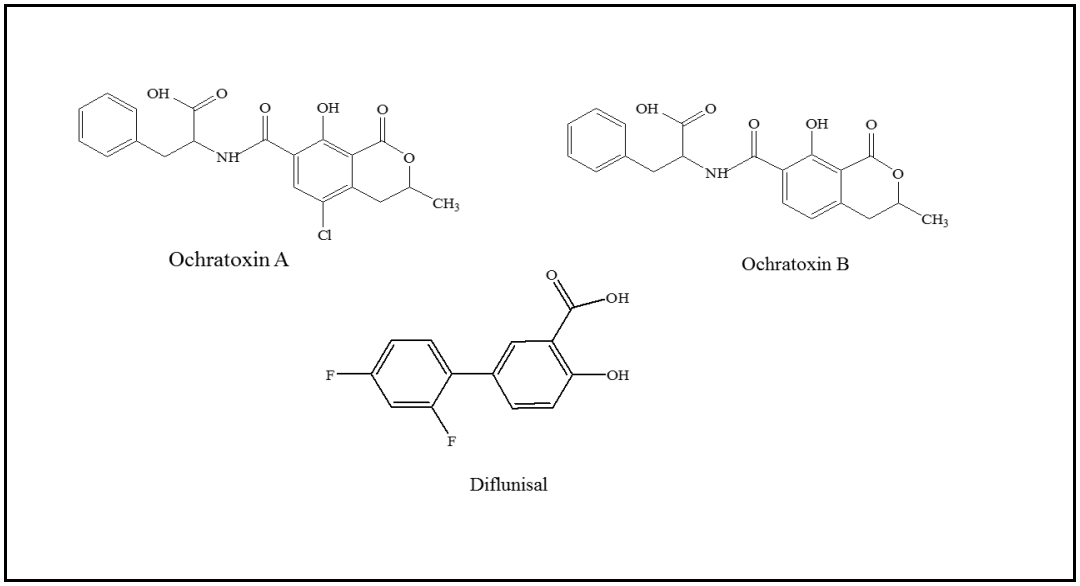


**Figure 1.Chemical structure of ochratoxin A, ochratoxin B and difunisal**

OTA and OTB can found in a large variety of foods including cereals, beans, spices, dried fruits, grapes, coffee and dried chili. It has also been detected in the milk, blood, liver, kidney and poultry meat from animals fed with OTA-contaminated feed[12-14].

In 2010, the European Union was set maximum limit for OTA at 15 ng/g including chilies, chili powder,dried chili, cayenne pepper, paprika, white and black pepper. were amended in Commission Regulation No. 105/2010 from July 2012.On thecontrary, there are no legal limits for OTB in food and dried chili, and they are not set in other regulations[15].

Dried chili is a commonly spices which ingredients in almost all popular dishes in Thailand, Malaysia and Indonesia. They are used for flavouring, seasoning and imparting aroma or colouring to foods. However, the raw materials of dried chili often originates from countries that lack adequate quality control and whose weather conditions during the growing season, along with improper harvesting and storage practices, can cause mycotoxin contamination[16].

Many analytical methods have been developed for quantifying OTA in various foodstuffs and animal feeds. Enzyme-linked immunosorbent assay (ELISA)and thin layer chromatography (TLC)have been developed to quantify and qualify OTA levels. However, there are disadvantage of the methods including less selective and metric effects in ELISA technique and low sensitivity in TLC. Rapid screening methods based on enzyme-linked immunosorbent assay (ELISA)[17] are easy to use; however, ELISA is less selective than other instrumental analysis methods and is prone to interference by sample matrices[18].

Liquid chromatography with fluorescence detection has been utilized for the determination of OTA at low concentration in foods[19,20]but, it requires cumbersome esterification steps[21]. Chromatographic methods with diverse detectors such as thin layer chromatography (TLC)[22], and high performance liquid chromatography with fluorescence detection(HPLC-FLD) which was used couple with immunoaffinity column cleanup (IAC) but the limitation of IAC is high cost[23] to analyze OTA in foodstuffs.

In the present study, we applied and validated method using liquid-liquid extraction followed by High performance liquid chromatography with fluorescence detection (HPLC-FD) to screen OTA and OTB in dried chili.

## 2. Materials and Methods

### Chemicals and reagents

Ochratoxin A, ochratoxin B and diflunisal were purchased from Sigma-Aldrich (St Luis, MO,USA).All chemicals were analytical reagent grade, except methanol, acetonitrile and ethyl acetate which were HPLC grade.

### Instruments

High performance liquid chromatography system consisted of The Agilent 1260 infinity Binary LC consisted of 1260 binary pump (G1312B), 1260 high performance Degasser (G4225A), 1260 high performance autosampler (G1367E), 1290 thermostatted column compartment (G1316C) and 1260 fluorescence detector (G1321B).

### HPLC-FLD Analysis

Ochratoxin A, ochratoxin B and difunisal were separated by Phenomenex Luna C18 column (150x4.60 mm 5 µm) and column oven was set up at 25 °C by modifying the method of Al-Hadithi[24].Isocreatic elution was employed with mobile phase consisting of Acetronitrile: 2% Acetic acid (60:40 V/V) with flow rate 0.7 ml/ min and injection volume was 5 µl. The fluorescence detector was set up by an excitation wavelength at 365 nm and an emission wavelength at 465 nm.

### Method validation

The developed chromatographic method was validated following AOAC guideline 2008.

### Linearity and range

Standard solutions of OTA and OTB were prepared in the methanol with concentrations 0.5, 1, 2, 5, 10, 20,30, 40, 50 and 100 ng/ml. Standard concentration of diflunisal used as an internal standard was 10, 20, 30, 40, 50, 100, 200 µg/ml .The concentration against peak response of each standard solution were used for the calibration curve. Linearity of relationships and good coefficients of determination ($R^2 \geq 0.9950$) were obtained.

### Limit of detection and limit of quantitation

Limit of detection (LOD) and Limit of quantitation (LOQ) were established by injecting seven times at 0.5 and 1 ng/ml for OTA and OTB and 80 µg/ml for diflunisal.LOD and LOQ were calculated according equation: LOD = 3SD and LOQ = 10SD, respectively.

### Accuracy

The accuracy of the method was determined %recovery using standard addition method. A known concentration of the OTA and OTB standard solutions were added into the chili powder which without contaminated with the OTA, OTB and another mycotoxins at low medium and high levels. The concentrations were analysed 5-times and calculated the % recovery.

### Precision

The precision was evaluated using Horwitzs ratio (HORRAT) in intra-day and inter-day. Various of the OAT, OTB and difunisal concentration was repeated 7 times for intra-day and on different 5 different days. The Horwitzs ratio was calculated according to equation:

Horwitzs ratio (HORRAT) = $0.66 \times 2 \times C^{-0.1505}$

When C is concentration ratio.

**Sample preparation**

65 samples of dried chili (32 samples for dried chili and 33 samples for dried chili powder) were purchased from local markets in Chiang Mai province, Thailand during March and April 2016.The amount of each samples were 100 g and were stored at 4 °C before analysis.

**Sample extraction**

25 grams of each sample was added with difunisal as internal standard and, the sample was extracted with ethyl acetate 3 times and filtered through Whatman No.1. The extracted solvent was evaporated. The residue was reconstituted with 1 ml ethyl acetate and filtrated through 2 µm nylon filter for HPLC analysis. The extraction was extracted in triplicate**.**

**Statistical analysis**

Data were expressed as the mean ± SD. Chi-Square test was used for comparing the mycotoxins in dried chili and dried chili powder.The levels of the OTA and OTB in the chili samples were compared using Wilcoxon matched- pared test and statistical significance was determined at $p<0.05$.

## 3. Results and Discussion

The HPLC-FLD chromatograms showed a good resolution for the OTA, OTB and difunisal and none of interference peakat the same resolution times. The retention time of the OTB, OTA and difunisal was 4.120, 5.729 and 7.780 mins, respectively. All of the analytes showed good linearity in concentration range 0.5-50 µg/L for OTA and OTB and 10-100 mg/L for difunisal with $R^2$ greater than 0.995 according to AOAC guideline 2008.

The LOD and LOQ of the analytical method of OTA were 0.25 and 0.5 µg/ L. While, LOD and LOQ of OTB were 0.75 and 0.9 µg/ L, respectively. This method showed highly quantified method with lower LOD and LOQ level than the other previous study and injection volume was only 5 µl.The results are shown in Table1.

**Table 1.The data of method validation**

| Substances | Linearity | | | LOD | LOQ |
|---|---|---|---|---|---|
| | Range | Equation | $R^2$ | | |
| OTA | 0.5-50 µg/ L | y=0.122x+0.022 | 0.9999 | 0.25 µg/ L | 0.50 µg/ L |
| OTB | 0.5-50 µg/ L | y=0.022x+0.014 | 0.9987 | 0.75 µg/ L | 0.90 µg/ L |
| Difunisal | 10-100 mg/ L | y=0.426+1.495 | 0.9976 | 8.0 mg/ L | 10.0 mg/ L |

The accuracy of the analytical methods was evaluated by percent of recovery. In experiment, OTA and OTB were spiked in 3 concentration (low, medium and high levels) according to Table 2. The spiked samples were extracted with ethyl acetate 3 times then the extract were analysed with HPLC-FLD.The percent recovery of the triplicate solutions was determined and average of the percent recovery was calculated. Acceptance of accuracy values are between 70-110 %.Riberiro and Alves determined OTA levels in grape pomaces using ethyl acetate extraction and showed % recovery of OTA which value was 23.5± 3.6%. Different types of sample including dry and wet sample may effect on % recovery[25].

Precision of the method was expressed in Horwitz's ratio (HORRAT) which HORRAT's value accepted less than 2 (AOAC, 2005). The repeatability was studied by repeating the assay seven times in the same day and intermediate precision was studied by repeating the assay on five different days, seven times on each day. The results are shown in Table 2.This method, the precision values were between 0.12 to 0.82 for OTA and OTB. Riberiro and Alves showed precision value 0.4-14.7%[25].

The current data showed the highest incidence of OTA and OTB in dried chili powder especially roased chili powder ($p<0.05$). Positive samples were analyzed the OTA and OTB levels and compared the levels. The result showed no significantly different of mycotoxin levels between dried chili and dried chili power.

Levels of OTA and OTB were found in range 0.10-0.66 ug/kg and 0.26 ug/kg, respectively. The results are shown in Table 3 and 4. All positive samples were below the EU maximum limit for OTA in spices. In present study, 5/16 of dried chili products (31.25%) contained OTA above the LOD of the amended analytical method, in range 508.28-2364.57 µg/kg. It can be concluded that the analyzed dried chili products have potential risk to consumers in Chiang Mai, Thailand. However, the positive samples should be confirmed with other condition or new sample preparation such as SPE or immune affinity column due to interfere of phytochemical in chili may give false positive.

**Table 2.The data of % recovery, repeatability and Horrat's ratio**

| **Mycotoxin (spiked levels)** | **Recovery (%)** | **Repeatability (%RSD)** | **Horrat's ratio Intra-day** | **Horrat's ratio Inter-day** |
|---|---|---|---|---|
| OTA(1.5 µg/ kg) | 93.79 | 1.01 | 0.50 | 0.45 |
| OTA(15 µg/ kg) | 93.10 | 1.41 | 0.12 | 0.11 |
| OTA(40 µg/ kg) | 88.26 | 0.77 | 0.15 | 0.08 |
| OTB (6 µg/ kg) | 94.86 | 1.20 | 0.83 | 0.79 |
| OTB (15 µg/ kg) | 92.78 | 0.76 | 0.74 | 0.40 |
| OTB (40 µg/ kg) | 87.06 | 0.76 | 0.82 | 0.45 |
| **Internal standard** | | | | |
| Difunisal (30 mg/ L) | 95.27 | 0.35 | 0.16 | 0.19 |
| Difunisal (50 mg/ L) | 94.04 | 0.75 | 0.12 | 0.14 |
| Difunisal (80 mg/ L) | 93.45 | 0.23 | 0.04 | 0.13 |

**Table 3 Ochratoxin A occurrence in dried chili and dried chili product**

| **Chili commodity** | **Amount of samples** | **Amount of samples contaminated with OTA level (%)** | | | **Range of contamination (µg/kg)** |
|---|---|---|---|---|---|
| | | **<LOD** | **LOD-LOQ** | **>LOQ** | |
| **Dried chili** | 32 | 29 | 2 | 1 | 0.10-0.66 |
| **Dried chili powder** | 31 | | | | |
| **Roasted** | 9 | 8 | 1 | ND | 0.26 |
| **Nonroasted** | 22 | 22 | ND | ND | - |
| **Dried chili product** | 16 | 11 | ND | 5 | 508.28-2,364.57 |

ND : Not detected

**Table 4 Ochratoxin B occurrence in dried chili and dried chili product**

| **Chili commodity** | **Amount of samples** | **Amount of samples contaminated with OTB level (%)** | | | **Range of contamination (µg/kg)** |
|---|---|---|---|---|---|
| | | **<LOD** | **LOD-LOQ** | **>LOQ** | |
| **Dried chili** | 32 | 32 | ND | ND | - |
| **Dried chili powder** | 31 | | | | |
| **Roasted** | 9 | 7 | ND | 2 | 3.6 – 4.65 |
| **Non roasted** | 22 | 22 | ND | ND | - |
| **Dried chili product** | 16 | 16 | ND | ND | - |

ND : Not detected

## Conclusion

The amended analytical methods are well-recognized and suitable for the analysis of OTA and OTB in dried chili and dried chili products, while parameters were within the acceptable range The occurrence of OTA and OTB shows that five dried chili products were above the levels permitted by the EU for safe consumption,

and chili products therefore have potential risk to consumers in Chiang Mai, Thailand. Surveillance should be done annually to minimize the risk of OTA and OTB in dried chili products.

## Acknowledgements

This research was supported by Faculty of Medicine Research Fund, Chiang Mai University, Chiang Mai, Thailand.

## References

1. Duarte SC, Pena A, Lino CM. A review on ochratoxin A occurrence and effects of processing of cereal and cereal derived food products. Food Microbiol., 2010, 27(2): 187-198.
2. El Khoury A, Atoui A. Ochratoxin A: general overview and actual molecular status. Toxins. 2010, 2(4): 461-493.
3. Geisen R, Mayer Z, Karolewiez A, Färber P. Development of a real time PCR system for determination of *Penicillium nordicum* and for monitoring ochratoxin A production in foods by targeting the ochratoxinpolyketide synthase gene. Syst. Appl. Microbiol., 2004, 27(4): 501-507.
4. Van Der Merwe KJ, Steyn PS, Fourie L, Scott DB, Theron JJ. Ochratoxin A, a toxic metabolite produced by *Aspergillus ochraceus* Wilh. Nature, 1965, 205(4976):1112-1113.
5. Sherif SO, Salama EE, Abdel-Wahhab MA. Mycotoxins and child health: the need for health risk assessment.Int J Hyg Environ Health, 2009, 212(4):347-368.
6. Creppy EE. Update of survey, regulation and toxic effects of mycotoxins in Europe. ToxicolLett., 2002, 127(1-3):19-28.
7. Schwerdt G, Bauer K, Gekle M, Silbernagl S. Accumulation of ochratoxin A in rat kidney in vivo and in cultivated renal epithelial cells in vitro. Toxicology, 1996, 114(3):177-185.
8. Bruinink A, Rasonyi T, Sidler C. Reduction of ochratoxinA toxicity by heat-induced epimerization. In vitro effects of ochratoxins on embryonic chick meningeal and other cell cultures. Toxicology,1997,118(2–3):205-210.
9. Mally A, Keim-Heusler H, Amberg A, Kurz M, Zepnik H, Mantle P, Völkel W, Hard GC, Dekant W. Biotransformation and nephrotoxicity of ochratoxin B in rats. Toxicol Appl Pharm. 2005, 206(1):43-53.
10. Ahn S, Lee S, Lee J, Kim B. Accurate determination of ochratoxin A in Korean fermented soybean paste by isotope dilution-liquid chromatography tandem mass spectrometry. Food Chem., 2016, 190: 368-373.
11. WHO. Evaluation of certain mycotoxins in food. Fifty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives.World Health Organ Tech Rep Ser, 2002, 906: 1-62.
12. Bascarán V, de Rojas AH, Chouciño P, Delgado T. Analysis of ochratoxinA in milk after direct immunoaffinity column clean-up by high-performance liquid chromatography with fluorescence detection. J Chromatogr A, 2007,1167(1):95-101.
13. Blesa J, Berrada H, Soriano JM, Moltó JC, Mañes J. Rapid determination of ochratoxin A in cereals and cereal products by liquid chromatography. J Chromatogr A, 2004,1046(1–2):127-131.
14. Visconti A, Pascale M, Centonze G. Determination of ochratoxin A in wine by means of immunoaffinity column clean-up and high-performance liquid chromatography. J Chromatogr A, 1999,864(1):89-101.
15. Jović SM, Petrović AV, Marković NR. Mycotoxins in wine with special attention on ochratoxin A. ZbornikMaticesrpskezaprirodnenauke, 2009,116:91-100.
16. Filazi A, Sireli UT. Occurrence of aflatoxins in food, INTECH Open Access Publisher, 2013.
17. Fukal L, Reisnerova H. Monitoring of aflatoxins and ochratoxin A in Czechoslovak human sera by immunoassay.B Environ ContamTox, 1990, 44(3):345-349.
18. Leitner A, Zöllner P, Paolillo A, Stroka J, Papadopoulou-Bouraoui A, Jaborek S, Anklam E, Lindner W. Comparison of methods for the determination of ochratoxin A in wine. Anal Chim Acta, 2002, 453(1):33-41.
19. Chan D, MacDonald SJ, Boughtflower V, Brereton P. Simultaneous determination of aflatoxins and ochratoxin A in food using a fully automated immunoaffinity column clean-up and liquid chromatography–fluorescence detection. J Chromatogr A, 2004,1059(1–2):13-16.

20. Sáez JM, Medina Á, Gimeno-Adelantado JV, Mateo R, Jiménez M. Comparison of different sample treatments for the analysis of ochratoxin A in must, wine and beer by liquid chromatography. J Chromatogr A, 2004,1029(1–2):125-133.
21. Lau BP, Scott PM, Lewis DA, Kanhere SR. Quantitative determination of ochratoxin A by liquid chromatography/electrospray tandem mass spectrometry. J Mass Spectrom, 2000, 35(1):23-32.
22. Scott PM, Lawrence JW, Van Walbeek W. Detection of mycotoxins by thin-layer chromatography: application to screening of fungal extracts. ApplMicrobiol, 1970, 20(5):839-842.
23. Dall'Asta C, Galaverna G, Dossena A, Marchelli R. Reversed-phase liquid chromatographic method for the determination of ochratoxinA in wine. J Chromatogr A, 2004, 1024(1):275-9.
24. Al-Hadithi N, Kössler P, Karlovsky P. Determination of ochratoxinA in wheat and maize by solid bar microextraction with liquid chromatography and fluorescence detection. Toxins (basel), 2015, 7(8): 3000- 3011.
25. Ribeiro E, Alves A. Comparative study of screening methodologies for ochratoxinA detection in winery by-products. Anal BioanalChem, 2008, 391(4): 1443-1450.

*****