Method Optimization and Determination of Ochratoxin A in Red Chilli (*Capsicum annuum* L.)

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Summary: Present study was planned to optimize a simple and rapid quantitative chromatographic method for the determination of OTA in red chilli. The validation of methodology was carried out by spikes recoveries, inter and intraday repeatability. Mean recoveries were 92.90%, 98.33%, 96.66 and 103.60 with repeatability RSD 10.85, 16.34, 5.95 and 9.82 percent respectively and regression coefficient was 0.999. The limit of detection (LOD) and limit of quantification (LOQ) were 0.5 ng/g and 1.0 ng/g respectively. For confirmation of the efficacy of optimized method, fifteen (n=15) samples of red chilies were randomly collected and subjected to OTA analysis. This random analysis revealed that eighty percent of samples were OTA contaminated, ranging from 23- 94 ng/g. Higher incidence with elevated levels of OTA in red chilli envisages an alarming situation as red chilli is an indispensible component of daily diet.

Key Words: Red chilli, OTA, immunoaffinity column,

Introduction

Ochratoxin A (OTA) is toxic fungal metabolite mainly produced by different species of fungi in different climatic conditions *i.e. Aspergillus* in warm and humid; *Penicillium* in cool and temperate regions [1]. Ochratoxin A is an analogue of alanine and well documented for its nephropatoxic, teratogenic and immunosuppressive effects in animals and human beings [2]. It can contaminate a wide variety of food, cereals, dried fruits and spices etc [3]. Among spices, red chilli (*Capsicum annuum* L.) of family *Solanaceae* is highly susceptible for fungal growth due to its hygroscopic nature [4]. In addition to hydroscopicitiy, extended exposure to environment during drying in open fields and poor storage practices invite fungal growth that ultimately leads to mycotoxins production particularly aflatoxins and ochratoxin A [5,6].

Red chillies (*Capsicum annuum* L) are dried ripe fruit of family *Solanaceae*. All over the world, it is extensively being used in traditional cuisines due to its pungent taste and appealing colour [7]. This crop is cultivated throughout the year in different climatic zone of Pakistan. Sindh province is the major production hub due to its warm and humid climate [8]. This warm and humid climate, in addition to its prolong drying in open fields and poor storage practices make it favorite substrate for the fungal growth particularly for the storage fungi Mycobiota of red chilli is extensively studied and documented [9]. It is highly contaminated with various pathogenic fungi predominately *Aspergillus*, *Penicillium*, *spp.* [7, 10, 11]. *Aspergillus and Penicillium* species are well known for the production of total aflatoxins and Ochratoxin A [12-13]. Aflatoxin contaminations in red chilies are well documented and extensively reviewed [5, 7, 11]. Despite of extensive data in the literature concerning the occurrence of OTA in different type of food *i.e.* coffee, corn, wheat, wine and rice but there is very limited data available for OTA contamination in spices, particularly in red chilies. This lacuna may be due inefficiency of conventional extraction methods as red chilli is highly complex and pigmented compound. This high level of colouration produce interference on HPLC trace masking the toxin results [14-16].

For incessant monitoring of OTA contamination in red chilli a simple, inexpensive, accurate, precise, sensitive and reproducible method is required.

In keeping view of foregoing, present study was planned to optimize a simple and reliable method for the determination of OTA in red chilies by using immunoaffinity clean up coupled with high performance thin layer chromatography (HPTLC). After optimization, randomly collected chilies samples collected from Rawalpindi were analyzed for OTA contamination.

Results and Discussion

Parameters of Method Optimizations

Method was optimized by evaluating parameters *i.e.* spike recoveries, inter and intra-day repeatability and regression equation. Fifteen samples of red chilies were randomly collected from market of Rawalpindi were analyzed for OTA residue.

Table-1: Spike recoveries with repeatability RSD values of Present HPTLC and HPLC method.

Spike Levels (ng/gm)	Present HPTLC Study				Lino *et al.*, 2006	
	*Developing Solution A (6:3:1; v/v/v)		**Developing solution B (18:1:1; v/v/v)			
	Mean Recoveries (%)	Repeatability RSD %	Mean Recoveries (%)	Repeatability RSD %	Mean Recoveries (%)	Repeatability RSD %
0.50	92.90[a]	10.85	91.04[a]	8.23	98.00[a]	10.0
1.00	98.33[b]	16.34	93.66[ab]	5.58	109.10[a]	9.90
2.00	96.66[a]	5.95	96.66[a]	10.64	102.30[a]	15.80
5.00	103.60[a]	9.12	105.62[a]	10.74	105.80[a]	7.50

Linearity

Calibration curve was drawn by using linear regression equation of expected versus observed OTA concentrations. Fig. 1 shows linear regression equation for OTA recovery for spiked red chilli samples in two different developing solutions from 0.25 to 64 ng. Linear regression equation showed good linearity as R^2 is 0.999 for solution A and 0.998 for solution B.

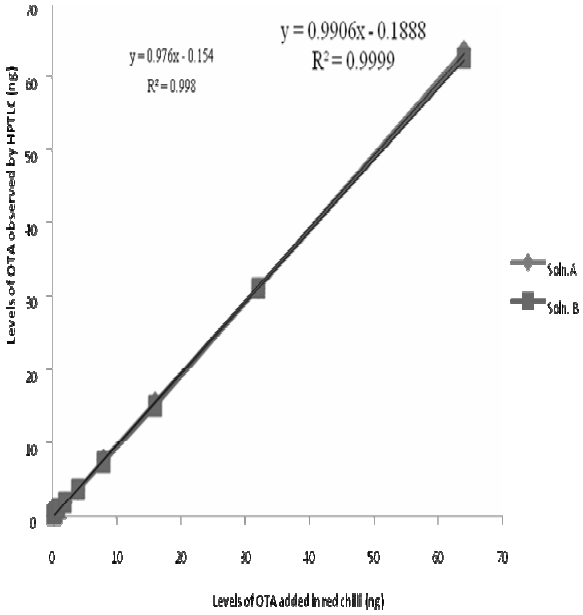

Fig 1: Linear regression equation for OTA spikes in developing solution A and B in the range of 0.25 to 64.

Accuracy

Four different spiking levels 0.5, 1.0, 2.0 and 4.0 was evaluated for accuracy. Mean spike recoveries were 92.90, 98.33, 96.66 and 103.6 percent with repeatability RSD values 10.85, 16.34, 5.95, and 9.12 percent respectively for spike samples developed in solution A. Similarly mean spike recoveries of OTA in red chilli were 91.40, 93.66, 96.66, 105.62 percent with RSD 8.23, 5.58, 10.60 and 10.74 percent respectively for spiked samples developed in solution B. All Spike recoveries were greater than eighty percent and comparable with standard /modified HPLC method. Results of spike recoveries in both developing solutions are non significant (*P<0.05).* Whereas, Spike recoveries for all fortified levels of present HPTLC method are non significant (P<0.05) except one fortified level *i.e.* 1 µg/mL (Table-1) with respect to modified HPLC method [15].

Precision

Precision was evaluated by intra-day and inter-day repeatability at fortification level 4.0 ng/g. For both developing solutions A and B, repeatability RSD values of intra and inter-day repeatability were 16.34 and 10.82 percent respectively. The limit of detection (LOD) was 0.5 ng/g and limit of quantification (LOQ) was 1 ng/g (Table-2).

Table-2: Accuracy and precision parameters of developing solutions A & B.

Parameters	Present HPTLC Study		Lino *et al.*, 2006
	Developing Solution A	Developing solution B	
Spike level (ppb)	4.0	4.0	5.00
Inter-day Repeatability (RSD)	16.34	10.82	7.5
Intra-day repeatability (RSD)	8.23	10.87	11.60
Limit of Detection (ppb)	0.5	0.5	0.05
Limit of Quantification (ppb)	1.0	1.0	0.10
R^2	0 .999	0.998	0.991

Method Confirmation

For the assessment of optimized method's efficiency, fifteen red chilli samples were randomly collected from local market and subjected to OTA analysis. Results of present study revealed that twelve (80%) samples were found positive for OTA ranging from 23 to 94 ppb in both developing solutions. Frequency distribution of contaminated samples further explained that no sample was found contaminated less than 20ppb. The highest frequency 38.46% lied in the range of 40-60 ppb followed by 15.38% (20-40, 60-80) and 7.6 % (80-100ppb) respectively (Fig. 2). Similar evidences of natural occurrence of OTA in red chili were also reported by some authors (Table-3).These elevated levels of OTA in red chilies samples might be due to prolong drying

and poor storage practices. In Pakistan, drying process involved spreading of one thick pods layer directly on soil, any sheet or on pavement [10]. By this practice, red chilies are sundried for 7-10 days [18]. During this exposure to environment, fungal spores from soil and air may inoculate into red chilli. Furthermore, its hygroscopic nature and prolonged storage in ware houses provide conducive environment for the germination of fungal spores resulting in mycotoxin production [12].

European Commission has legislated 10ppb of aflatoxin in red chilli but no regulatory limits for OTA [14], which has been declared as Class 2B by International Agency for Research on Cancer [6]. It may be due to unavailability of sufficient data. These high levels of OTA in chilli samples are alarming as it is indispensable part of daily diet of all over the world predominantly in Asian countries. Such high OTA levels might be considered as contributing factor for the increase rate of nephrotoxicity/ kidney diseases in country as OTA is well documented for its nephrotoxic effects [18-20].

Table-3: A scenario of Ochratoxin A contamination in red chilli.

Total samples (n)	Positive (%)	Min. – Max. (ppb)	Reference
15	80	23-94	Present Study
70	85.70	0.24-93.0	Shundo *et al.*, 2009
70	45.71	10.6-66.2	Fazekas *et al.*,2005

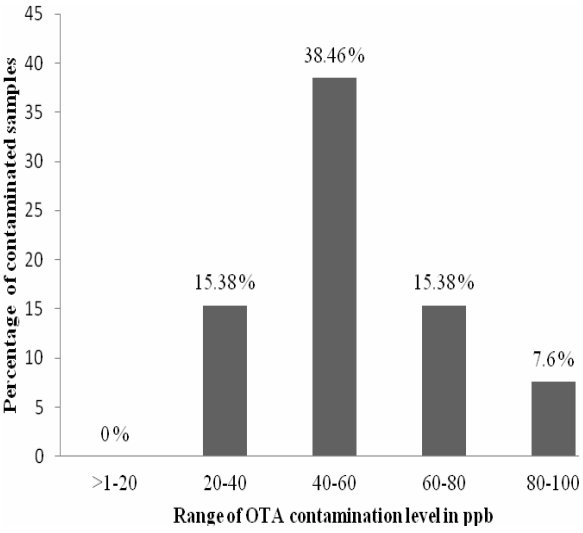

Fig. 2: Frequency distribution of OTA contamination in red chilli samples.

Experimental

A certified standard solution of Ochratoxin A having concentration 10.05 µg/kg was purchased from Biopure (Austria). One stock solution was prepared in 5ml toluene-acetic acid (99:1; v/v) at 1.005 µg/mL. Intermediate solution was prepared at 0.1005 µg/mL, by diluting 1ml of stock solution with 10 mL of toluene-acetic acid (99:1; v/v). For fortification assays, working solutions were prepared in toluene: acetic acid (99:1; v/v) at 0.01 005 µg/mL. All solutions were kept in amber vials to protect from light.

Extraction and Cleanup

A method described by [15] for OTA analysis by immunoaffinity column (IAC) coupled with HPLC was modified and optimized. A 12.50 gm ground red chilli samples were homogenized with a 50 mL methanol: water (80:20; v/v) blend for 3 minutes and filtered through Whatman filter paper No. 1. A 44 mL of phosphate buffer was added in 4 mL of sample extract and refiltered to remove fat layer and pH was adjusted to 7.4 by using 0.1M NaOH or HCl as required. After pH adjustment sample extract was loaded on the OchraStar^{TM} IAC (Romer, Austria) at flow rate 2 ml/min. On completion of loading, column was washed with 30 mL distilled water (until antibody gel free from pigments) at a flow rate 3ml/min then OTA was eluted with 2 mL of methanol at 0.5 ml/min. The eluant was dried at 60 ºC. redissolve in 400 µl of toluene: acetic acid (99:1; v/v). A volume of 100 µl redissolve sample was spotted on two HPTLC plates along with different concentration of OTA standard. The plates were develop in two different developing solutions *i.e.* solution A (toluene: acetic acid: formic acid cid; 6:3:1; v/v/v) and solution B (toluene: methanol: acetic acid; 18:1:1; v/v/v). The R_f of OTA was 0.6 and 0.2 for solution A and B respectively. After developing, OTA was estimated under UV light (365nm) by comparing size, intensity and Rf of spike spots with standard spots.

Statistical Analysis

Significance of spike recoveries was calculated by analysis of variance (ANOVA) and least significance difference (LSD) by using MSTAT software.

Conclusion

The findings of present study illustrated good results in term of sensitivity, accuracy, inter and intra-day precision. Furthermore, >80% recoveries showed that both developing solutions A and B are suitable for separation of OTA on HPTLC technique. These recoveries are comparable with HPLC method as described by [15]. The results of present study

revealed OTA continue to pose a health concern via human exposure to contaminated red chilli. There is dare need to set regulatory limits for OTA in red chilies to ensure the availability of OTA free chilies to final consumer. Present optimized HPTLC method can be used for the conduction of large scale survey to assess the exact scenario of contamination of OTA and to establish a regulator limits in red chilies.

References

1. B. Zimmerli and R. Dick. *Journal of Chromatography B: Technology*, *Biomedical*, **666**, 85 (1995).
2. E. E. Creppy, *Toxicology Letters*, **127**, 19 (2002).
3. International Agency for Research on Cancer – IARC, Monograph on the Evaluation of Carcinogenic Risks to human. Some Naturally Occurring Substances: Food items and constituents, Heterocyclic Aromatic Amines and Mycotoxins. Lyon 56 (1993).
4. M. Banerjee, and P. K. Sarkar, *Micro Food Research International*, **36**, 469 (2003).
5. H. Colak, E. B. Bingol, H. Hampikyan, B. Nazli, *Journal Food and Drug Analysis*, **14**, 292 (2006).
6. Q. A. Mandeel, *Journal of Mycopathololog*y, **59**, 291 (2005).
7. Z. A. Shamsudddin,, A. K. Mobeen, A. K. Butool, A. Mansoor, A. Aftab. *Journal of Mycotoxin* Research, **11**, 21 (1995).
8. PARC, National Master Agricultural Research Plan. PARC, MINFAL, Islamabad, Pakistan, (2006).
9. S. I. Shamshad, R. B. Zuberi. *Pakistan Journal of Scientific and Industrial Research*, **28**, 395 (1985).
10. R.D. Kiran, K. J. P. Narayana, M. Vijaylakshmi, *African Journal Biotechnology*, **4**, 791 (2005).
11. R. Russell, M. Paterson. *Journal of Food Control*, **18**, 817 (2007).
12. C. M, Lino, B. Lurdes, S. P. I. N. S. Angelina. Journal of Quimica. Nova. 29, (3): 436 (2006).
13. A. Hussain, J. Ali, Shafqatullah, *Journal of the Chemical Society of Pakistan*, **33**, 481,(2011).
14. B. J. Abbas, M. Arif, F. Khan, *Journal of the Chemical Society of Pakistan*, **33,** 275 (2011).
15. S. H. Cho, C. H. Lee, M. R. Jang , S. M. Lee, L. S. Choi, *Journal of Food Chemistry*, **107**, 699 (2008).
16. A. Hussain, J. Ali and Shafqatullah, *Journal of the Chemical Society of Pakistan*, **33**, 481 (2011).
17. Commission Regulation (EC). *Official journal of European Community*. **34**, 1881 (2006).
18. Kuiper-Goodman, P. M. Scott. *Biomedical Environmental Sciences*, **2**, 179 (1989).
19. L.Shundo, A. P. D. Almeida, J. Alaburda, L. C. A. Lamardo, S. A. Navas, V. Ruvieri, M. Sabino. *Brazilian Journal of Microbiology*. **20**, 1099 (2009*)*.
20. B. Fazekas, A. Tar, M. Kovacs. *Journal Food Additive and Contaminant*, **22**, 856 (2005).