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Analytical method for determination Ochratoxine A in some kinds of food

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Abstract: Ochratoxine A(OTA) is a widespread and abundant natural carcinogenic mycotoxin produced by several species of Aspergillus and Penicillium fungi. Due to the ubiquitous presence of these fungi in food and potential risk for human health, a rapid and sensitive in vitro detection assay is required. For routine testing of ochratoxin A (OTA) in cereal feed samples, a reverse-phase high-performance liquid chromatographic fluorescence detection (HPLC-FLD method, with immune affinity column cleanup was developed.
Keywords: Ochratoxine A, System Precision, Repeatability, HPLC-FLD.

Introduction:

Nowadays, researchers focus increasingly on common food contaminants like mycotoxins. These ubiquitous toxins are produced by the secondary metabolism of fungi, mainly saprophytic molds. More than 400 mycotoxins, with different chemical structures and toxic effects, have been identified[1]. Among them, ochratoxin A (OTA) has attracted much attention because of its abundance and toxicity. OTA is produced by two main genera of filamentous fungi, *Aspergillus* and *Penicillium*, which grow on a variety of food products[2]. Structurally, OTA consists of a chlorinated dihydroisocoumarin moiety linked with a 7-carboxyl group to L-β-phenylalanine[3]. Therefore, it has an inhibitory effect on a number of enzymes that use phenylalanine as substrate. It is a mitochondrial poison, causing mitochondrial damage, oxidative burst, lipid peroxidation, and interferes with oxidative phosphorylation[4,5]. Moreover, reducing OTA levels from foodstuffs is not feasible because it is very resistant to food preparation procedures such as cooking, roasting, and fermenting[6]. The presence of OTA in food samples at very low concentration may induce toxic effects, therefore selective and sensitive detection of OTA is highly required in order to guarantee food safety and to minimize the potential risk to human and environmental health. Due to its fluorescent nature, OTA is generally determined by chromatographic techniques[7].

Ochratoxin genesis can occur during crop growth, harvest, storage, or processing, which may lead to OTA contamination in several food and feedstuffs. This mycotoxin is widely found in cereals such as corn, wheat, barley, oats, and millet and cereal derived products such as flour, bread, beer, and vodka[8-11]. After cereals, wine is considered as the second source of human consumption (10% to 15% of daily OTA intake)[12,13]. Recently, the contamination of wines and grape juices by OTA has been shown in different studies[14]. OTA has also been identified as a contaminant of coffee beans, cocoa, dried fruits, and spices throughout the world. Moreover, OTA is a natural contaminant of farm animal feeds and it has been traced in meat, milk, and dairy products. As a widespread food contaminant, OTA was found in human blood, milk, and urine. This mycotoxin has several effects on animal and human health. The main harmful effect of OTA is its nephrotoxicity, as demonstrated by many studies on animals. Additionally, OTA causes acute and chronic nephropathies to

humans, and it is suspected to be the main etiological agent responsible for Balkan Endemic Nephropathy (NEB), a chronic tubulointerstitial renal disease. Based on the carcinogenic potency of OTA to rodents, the international Agency of Research on Cancer (IARC) has considered it as a potential carcinogen for humans (group 2B). Immune toxic and myelotoxic effects of OTA have been also reported. The immunosuppressant activity of OTA is characterized by alteration in number and activity of immune cells and size reduction of immune organs. In addition, OTA induces diverse effects on hematopoietic progenitor proliferation. Finally, OTA has been observed to be teratogenic with diverse fetus malformations in a number of animal models[15].

Based on the toxicological evaluation performed by scientific committee on food, the European Union has established a tolerable weekly intake of 120 ng of OTA per kg of body weight (Commission Regulation No. 594/2012)[16]. In addition, some directives have been introduced by the European Union in order to set the maximum permitted levels of OTA in foodstuffs such as cereals (5 µg/kg) and all cereal-derived products (3µg/kg), roasted coffee and coffee products (5µg/kg), grape juice and all types of wine (2 µg/L), dried fruits (10µg/kg), and spices (15 µg/kg) (Commission Regulation No. 123/2005). Furthermore, the maximum tolerated amounts of OTA have been fixed in cereals used in the composition of feedstuffs (0.25 mg/kg), the complementary and complete feed for pork (0.05 mg/kg), and poultry (0.1 mg/kg) (Commission Regulation No.576/2006)[17]. Moreover, in a Canadian study performed on various sex/age groups, a virtually safety dose of 4 ng/Kg bw perday was calculated[18]. Nowadays, the most widely used quality control process relies on an immune affinity column (IAC), followed by reversed-phase high pressure liquid chromatography using fluorescence detection (HPLC-FLD)[19,20]. IN this paper, OTA was determined in some kinds of flour and rice used in local markets in Syria. The paper "contributions to the analytical study of the Ochratoxines with applications on food products" intends to establish and validate methods for determining Ochratoxine A by high performance liquid chromatography method and their implementation on various products of vegetal origin and also identify the level of contamination.

.2-Experimental Procedure:

2.1-Materials:

Acetonitrile and methanol (HPLC-gradient grade) were obtained from Sigma-Aldrich (Germany). Standard OTA were obtained from Sigma-Aldrich (Germany). Potassium dihydrogen phosphate from Merck(Germany).,potassium chloride from panreac (spin), sodium chloride from panreac (spin). Some kinds of flour and rice were broached from local markets. Immune affinity columns for Ochratoxine A were broached from Germany.

2.2-Apparatus:

The High Performance Liquid Chromatographic (Shimadzu, Japan, Kyoto). The device was supplied with; pump model(LC-20AT), the oven model (CTO- 20A); OTA Was completely separated using a stainless steel column of dimension (4.6 × 250 mm^2) packed with symmetry C_{18} and 4 µm particle size (Merck , Germany). The detection system model (RF10AXL), the signal acquired from detector was recorded by a personal computer operated by using LC solution program. Auto sampler (SIL20A) HPLC (Hamilton- Bonduz, Schweiz, Switzerland, 100µl).

2.3-Chromatographic Conditions:

The chromatographic experiments for determine OTA were carried out by using HPLC-FLD as follows; Mobile phase: Acetonitrile, water and acetic acid (60:39:1)., *column oven temperature:40°C., *injection volume: 50 µl., *flow rate: 1ml/min *detection fluorescence: (excitation at 333nm,emission at 460 nm).

2.4- preparation of Standard Solution:

Individual stock standard solution of OTA (1000 mg/L) was prepared in methanol, then, this stock solution was diluted with methanol to obtain samples solutions with concentrations ranging from 20ppb to 100ppb.

2.5- Extraction of Ochratoxine A from Samples:

Ochratoxine A was extracted from 10g of sample by using 40ml methanol/water (80/20 v/v). The extract was filtered, then 4ml of filtered extract were diluted to 50ml with phosphate-buffered saline (PBS), and

applied to an Ochratoxine A immune affinity column. The column was washed with water and the Ochratoxine A was eluted from immune affinity column with methanol.

3- Method Validation and Results:

Checking of certain parameters of validation was performed accordance to articles[21,22].

3.1- Selectivity:

Method selectivity was demonstrated for type of application on different categories of sample and showed that there are no other interfering peaks at retention time of Ochratoxine A.

3.2-Specificity:

In order to study the specificity of the method, Ochratoxine A was identified by injection a solution of a known concentration (40ppb) in methanol. For the OTA standard solution the retention time is 5.880 minutes (Figure-1,2). Tests have also been carried on the solvent used methanol in the preparation of standard solutions and samples. To the retention time corresponding to Ochratoxine A no interfering peaks were obtained.

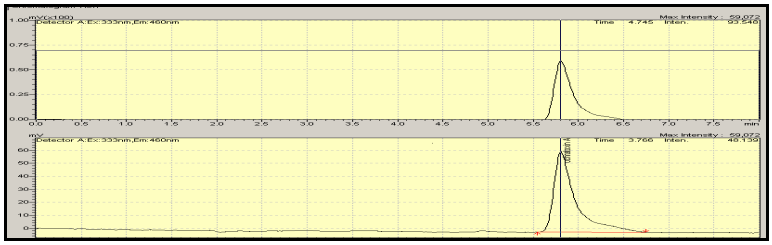

Figure -1:the chromatogram for OTA- standard solution.

Figure -2:the chromatogram for OTA- in wheat sample

3.3-Linearity:

Standard solution of OTA were prepared and analyzed, with concentrations ranging from 20ppb to 100ppb. Figure-3, shows the regression line obtained in the linearity of the method for determining the OTA in working area.

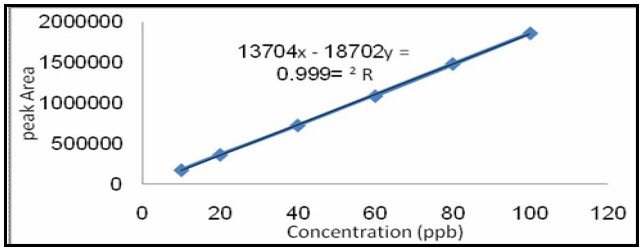

Figure- 3: Linearity line

3.4-The limit of detection and the limit of quantification:

For determining the limit of detection and the lower limit of quantification was used the method of calculation using S/N:

$$LOD = 3 \times \frac{S}{N} = 3 \times \frac{95}{18755} = 0.015 ppb$$

$$LOQ = 10 \times \frac{S}{N} = 10 \times \frac{95}{18755} = 0.051 ppb$$

3.5- Precision:

In order to examine the accuracy, several degrees of precision have been tested: the system precision, method precision, and intermediate precision.

A- System Precision (Injection repeatability):

To examine the precision of the system, the same solution containing Ochratoxine A in the concentration of interest (40ppb) was injected 6 times resulting the same number of chromatograms. From the chromatograms obtained the peak areas were measured, calculating the mean area, standard deviation and relative standard deviation. The obtained experimental values are illustrated in table-1. From the results., shown in table-1, it could be notice that the determination of OTA by HPLC is accurate because the obtained value of relative standard deviation (RSD = 0.7512%) lower than admitted limit (5%).

Table-1: Values of System Precision

Number of determination	Peak Area
1	71.8570
2	73.2230
3	72.9197
4	71.9999
5	72.8999
6	72.0999
Average (mean area)	72.4999
SD	0.544619
RSD	0.7512

B- Repeatability (Method Precision):

To establish the repeatability of the method for determining OTA, it was chosen to work with9 determination covering the specific areas of concentration (3 determinations at three concentration levels).According to experiment data (that was illustrated in table-2), the method is accurate, because the relative standard deviation (RSD = 0.841959%) lower than admitted limit (5%).

Table-2:Values of Method Precision

NO. Number of determination	Theoretical Concentration (ppb)	Peak Area	Calculated Concentration (ppb)	Recovery%
1	40	72.8999	39.71249	99.28123
2	40	71.9999	39.23123	98.07807
3	40	73.2230	39.88525	99.71312
4	60	109.0499	59.04197	98.40323
5	60	107.9653	58.46204	97.43673
6	60	107.9879	58.47412	97.45687
7	80	148.6961	80.02408	100.0301
8	80	146.9989	79.33339	99.16674
9	80	146.7996	79.22682	99.03353
Statistical Data			Average	98.58787
			SD	0.83007
			RSD	0.841959

C- Intermediate Precision:

To determine Ochratoxine A by HPLC, the intermediate precision was determined in different three days, the obtained experimental values are illustrated in table-3.According to experiment data, the method is accurate, because the relative standard deviation (RSD = 0.96838%) lower than admitted limit (5%).

Table-3:Values of Intermediate Precision

Number of determination	Theoretical Concentration (ppb)	Peak Area	Calculated Concentration (ppb)	Recovery%
1 first day	40	72.0786	39.27330	98.18330
2 second day	40	71.8987	39.17711	97.94280
3 third day	40	72.8999	39.71249	99.28123
1 first day	60	107.9757	58.46759	97.44591
2 second day	60	108.9998	59.01519	98.35860
3 third day	60	109.5238	59.29536	98.82560
1 first day	80	148.6961	80.02408	100.03010
2 second day	80	148.0768	79.90974	99.88720
3 third day	80	149.3462	80.5884	100.73550
Statistical Data			Average	99.21382
			SD	0.96077
			RSD	0.96838

3.6: Determination of Ochratoxine A in food samples (flour and rice)

Seven different samples of flour and six different samples of rice were analyzed by using HPLC to determine Ochratoxine A. The identification of the peaks corresponding to Ochratoxine A was made by comparison of their retention time with corresponding peak in the standard solution. The values of Ochratoxine A in food samples presented in Table 4.

Table-4: Values of Ochratoxine A in some food samples.

Number of flour sample	Value of OTA (ppb)	Number of rice sample	Value of OTA (ppb)
S1	Absent*	S1	o.o7
S2	Absent*	S2	Absent*
S3	0.08	S3	Absent*
S4	Absent*	S4	Absent*
S5	0.12	S5	Absent*
S6	Absent*	S6	0.08
S7	Absent*	-	

*- under LOD of method.

4-Conclusions:

The above-described HPLC method for determination of Ochratoxine A in some food samples provides a precision of the results of OTA content. The method is easy to apply in routine laboratory practice.

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References

1. Meerdink G.L. Mycotoxins. Clin. Tech. Equine Pract. 2002;1:89–93.
2. Fernández-Cruz M.L., Mansilla M.L., Tadeo J.L. Mycotoxins in fruits and their processed products: Analysis,occurrence and health implications. J. Adv. Res. 2010;1:113–122.
3. Van der Merwe K.S., Steyn P.S., Fourie L., DeScott B., Theron J.J. Ochratoxin A, a toxic metabolite produced by aspergillus ochraceus. Nature. 1965;205:1112–1113.
4. International Agency for Research on Cancer (IARC) Some Naturally Occurring Substances: Food Items andConstituents, Heterocyclic Aromatic Amines and Mycotoxins. World Health Organization; Geneva, Switzerland:1993.
5. Monographs on the Evaluation of Carcinogenic Risks to Humans. International Agency for Research on Cancer; Lyon, France: 1995. 489–521.
6. Boudra H., Le Bars P., Le Bars J. Thermostability of ochratoxin a in wheat under two moisture conditions. Appl. Environ. Microbiol. 1995;61:1156–1158.
7. Turner N.W., Subrahmanyam S., Piletsky S.A. Analytical methods for determination of mycotoxins: A review.Anal. Chim. Acta. 2009;632:168–180.
8. Duarte S.C., Pena A., Lino C.M. A review on ochratoxin A occurrence and effects of processing of cereal and cereal derived food products. Foodmicrobiol. 2010;27:187–198.
9. Ozden S., Akdeniz A.S., Alpertunga B. Occurrence of ochratoxin A in cereal-derived food products commonly consumed in turkey. Food Control. 2012;25:69–74.
10. Araguás C., González-Peñas E., López de Cerain A. Study on ochratoxin a in cereal-derived products from spain. Food Chem. 2005;92:459–464.
11. Tozlovanu M., Pfohl-Leszkowicz A. Ochratoxina in roasted coffee from french supermarkets and transfer in coffee beverages: Comparison of analysis methods. Toxins. 2010;2:1928–1942.
12. Cabañes F.J., Accensi F., Bragulat M.R., Abarca M.L., Castellá G., Minguez S., Pons A. What is the source of ochratoxin a in wine? Int. J. Food Microbiol. 2002;79:213–215.
13. Mateo R., Medina Á., Mateo E.M., Mateo F., Jiménez M. An overview of ochratoxin A in beer and wine. Int. J. Food Microbiol. 2007;119:79–83.
14. Bazin I, Andreotti N, IbnHadjHassine A, DeWaard M,.Sabatier JM, Gonzalez C. Peptide binding to ochratoxin A mycotoxin: A new approach in conception of biosensors. Biosensors and Bioelectronics Journal. 2013; 40; 240–246.
15. Rhouati A, Yang C, Hayat A, Marty J L. Aptamers: A Promosing Tool for Ochratoxin A Detection in Food Analysis. Toxins J. 2013; 5(11): 1988–2008.
16. Commission of the European Communities Commission Recommendation (EC) No 594/2012 of 5 July 2012emending regulation 1881/2006 as regards the maximum levels of the contaminants Ochratoxin A, non dioxin-like pcbs and melamine in foodstuffs. Off. J. Eur. Commun. 2012;L176:43–45.
17. Commission of the European Communities Commission Recommendation (EC) No 576/2006 of 17 August2006 on the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2 and fumonisins in products intended for animal feeding. Off. J. Eur. Commun. 2006;L229:7–9.
18. Kuiper-Goodman T, Hilts C, Billiard S M, Kiparissis Y, Richard I D k, Hayward S. Health risk assessment of ochratoxin A for all age-sex strata in a market economy. Food Addit. Contam. Part A. 2009;27:212–240.
19. Visconti A, Pascale M, Centonze G. Journal of Chromatography A. 1999; 864, 89–101.
20. Aresta A, VatinnoR , Palmisano F, Zambonin C G. 2006. Journal of Chromato- graphy A 1115, 196–201.
21. Guideline for samples and analytical data for methods validation, FDA,1987.
22. Oprean R, Rozet E, Dewé W, Boulanger Bm Hubert P H. Ghid de validare a proceduriloranaliticecantitativ, ClujNapoca: Ed. Medicalãuniversitarã Iuliu Haţieganu. 2007.

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