The Occurrence of Ochratoxin A in Hard Wheat and its Products During Milling Processes followed by Certain Mills in Tripoli

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ABSTRACT

This research aimed at detecting the presence of Ochratoxin A (OTA) in local hard wheat and tracking its traces in semolina produce during milling processes followed in some mills in the area of Tripoli. 96 samples of wheat and its derivatives are collected from three mills (32 sample each) at the four stages of the milling process: wheat storage, purification and cleaning, semolina production, and bran separation. A solid phase extraction method is used to obtain the poison (RPSPE 18) and to clean the extract. A high-performance liquid chromatography (HPLC) is employed to detect OTA and estimate its levels. The limits of quantifications were determined to be 0.025 ppb and the recovery rate to be 94%. The results showed that 88% (21/24) of the uncleaned wheat samples are contaminated with OTA, with concentrations ranging from 1.4-11.8 and an average value of 5.9 µg/kg. The percentage of contamination in cleaned wheat reached 79% (19/24), with concentrations ranging from 1.4 to 9.3 µg/kg and an average concentration of 5.3 µg/kg. Toxin's presence in semolina decreased by almost 50% (12/24) compared to raw wheat. The concentration observed in semolina ranged between 0.6 and 2.1 µg/kg, with an average of 1.2 µg/kg. On the other hand, the percentage of contaminated bran samples were 83% (20/24), with concentrations varying from 0.7 to 6.9 µg/kg and an average of 4.2 µg/kg. The results showed that 88% of raw wheat samples were of average OTA concentration slightly above the limit of 5 µg/kg as set by the Libyan standard No. 231/2005. On the other hand, the average OTA concentration in all semolina samples was below the limit of 3 µg/kg set for this product in the same standard. Milling processes led to a significant reduction (38%) of the presence of OTA in semolina. The average concentration of the toxin in the semolina also dropped by approximately 80%. The (ANOVA) analysis showed significant variations in OTA presence between each of the raw, cleaned wheat, and semolina/bran (C.L of 99%).

تواجد الاكراتوكسين أ في القمح الصلب ونواتجه تحت عمليات الطحن المتبعة ببعض المطاحن بطرابلس

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المُستلخص

تم تجميع 96 عينة من القمح الصلب ومشتقاته (سميد وردة) من 3 مطاحن تقع بطرابلس (32 عينة من كل مطحن) عبر اربع مراحل لعملية الطحن: خزن القمح الخام: الغربلة والتنظيف: استخلاص السميد وفصل النخالة. استخدمت طريقة الاستخلاص بالطور الصلب لاستخلاص وفصل السم كمااستخدم جهاز الكروتاموجرافي السائل عالي الأداء للكشف عن السم وتقدير مستوياته. بلغت الحدود الكمية التي أمكن قياسها (0.025) ومعدل الاسترجاع 94%. بينت النتائج بأن 88% (21/24) من عينات القمح الخام (غير المنظف) كانت ملوثة بهدا المركب وبمدى ومتوسط تركيز بين 1.4و 11.8 و 5.9 ميكرو جرام/ كيلو جرام على التوالي. بعد تنظيف تراجعت كل من نسبة التلوث في القمح الى 79%(19/24) ومدى التركيز مابين 1.4- 9.3 ميكروجرام/ كيلوجرام ومتوسط التركيز الى 5.3 ميكروجرام/كيلوجرام. كما اشارة نتائج تحليل عينات السميد الى انخفاض كل من: مستوى التلوث بهدا الناتج الى 50% (12/24) ، مدى تركيز السم (الذي تراوح بين 0.6 الى 2.1 ميكروجرام/ كجم) ومتوسط تركيزه الذي بلغ 1.2 ميكروجرام/كيلو جرام. من جانب اخر، ارتفع نسبيا مستوى تلوث الردة الى 83% (20/24) وتراوح تركيز الاكراتوكسين أ بها مابين 0.7 و6.9 ميكروجرام/ كيلوجرام وسجل متوسط التركيز 4.2 ميكروجرام/ كيلو جرام. متوسط تركيز الاكراتوكسين أ في معظم عينات القمح الخام (21/24 او 88%) كانت اعلى قليلا من الحد (5 ميكروجرام/كجم)المنصوص عليه بالمواصفة القياسية الليبية رقم 231/2005 لسميد القمح. بالمقابل فمتوسط تركيز هذا المركب في السميد كان اقل من الحد المقرر(0.3 ميكروجرام/كيلو جرام) له بنفس المواصفة. ادت عمليات انتاج السميد الى انخفاظ معنوي (38%) لتواجد الاكراتوكسين أ في هذا الناتج (من 88% الى 50%) كما تراجع متوسط التركيز به بنسبة 80% (من 5.9 الى 1.2 ميكروجرام/ كيلو جرام) عما كان عليه بالقمح الخام. أوضح التحليل الإحصائي بوجود تباين معنوي في تواجد الاكراتوكسين أ بين كل من: القمح الخام/ القمح المنظف/ السميد والنخالة عند حدود ثقة 99%. يستخلص الى ضرورة وأهمية التقيد بالممارسات الجيدة في تداول وتخزين القمح.

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الكلمات الدالة

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Introduction

Grain crops are considered one of the main ingredients included in most peoples' diets as they are the cheapest source of energy in human nutrition. Grains supply a person with about 1/3 of her energy and protein needs; they represent 60% of protein sources in the world. Wheat accounts for 33% of the total quantities of grain produced in the world (Abd al-Saeed, 1983; Hawass, 1994). However, wheat grains are exposed to different fungi, starting from the time of their physiological maturity until their final consumption or agriculture usage. There are around 100 different fungus species that can accompany grains, among which certain constitute major risks to grain-based plants, such as signs of deformities and colorations, or the appearance of black dots on the grains owing to certain fungi in the field when the humidity is higher than 90%. Other fungi cause decay and toxins exertion during storage, which poses health risks to consumers. Indeed, mycotoxins are considered one of the most important food contaminants. They incited great interest by many countries and organisations since they endanger public health and are behind many chronic diseases (Elshabal, 2004). The most important mycotoxin contaminating wheat are Aflatoxins, all kinds of Ochratoxins, Zearalenone, and Fumonisins, among others. However, the most important among these is Ochratoxin A (OTA), which is the result of a metabolic by-product of the fungi species Aspergillus and Penicillium. These species are especially found in grains, thus representing 55% of the amount of OTA consumed in some European countries according to statistics reported in the Official Journal of European Communities in 2006. It has been proven by many studies that these mycotoxins are related to many cases of poisoning in the world. Moreover, OTA is considered one of the leading pollutants that require extensive examination and proper estimation. Detecting and estimating OTA became more urgent after its proven association to the incident of Balkan countries, histologically called Balkan Nephropathy; and to the swine incident in Denmark in 1950, which resulted in the disruption of the urinary system of these animals due to consuming contaminated feed with high levels of OTA. Different studies have reported different percentages of OTA in urine, blood, milk, and tissues depending on food consumed by subjects. These results are used as biological indicators of human exposure to OTA (Ghali, 2009). Hard wheat is an important commodity for a number of processing units, being a primary ingredient for semolina production; the major material making up; pastry, couscous, soups etc. The semolina demand by the above processing units is provided by hard wheat milling undertaken by a number of milling plants. In 2003, for instance, the total semolina production was 150,000 tons (El-zayde, et al. 2003). By 2014, the total targeted production capacity was 700 thousand tons (JSNCMF, 2014). The storage and subsequent handling of such huge raw material is supposed to be in proper silos under well controlled conditions, which is hard to satisfy locally, all at once. Locally produced hard wheat is more likely stored and handled in a bad conditions, particularly in remote areas in southern Libya, thereby exposing this commodity to health threatening fungi growth. Therefore, and since data on contamination of the products and derivatives with this toxic compound and its fate under local milling conditions is lacking (as far as we know), the present study aims to detect the occurrence of OTA and its possible residue in hard wheat and its derivatives, respectively, and to compare contamination levels to allowable limits.

Materials and Methods:

Chemicals

The OTA solution with a concentration of 10µg/kg was supplied by sigma Aldrich Company. The operational solutions were prepared by mitigating the solution with methanol, acetonitrile, and 85% phosphoric acid. The toxin is isolated using solid-phase extraction, where sodium hydrogen bicarbonate solution (1% weight/ volume), phosphate buffered saline (PBS), water, and methanol are used to wash the columns (SPE, RP18). (AOAC International, 2004).

Sample collection

96 samples of raw wheat grains and their produce were collected from three mills in Tripoli, all of which are subsidiaries of the National Company for Mills and Fodder. 32 samples were taken from each mill at all four stages of the milling process: raw wheat storage, purification, and cleaning of wheat, semolina production, and bran separation. Specimens were collected between November 2009 and January 2010. The moisture content in raw wheat was measured simultaneously and its levels ranged from 6-11%. The moisture content in wheat after being cleaned and before milled into semolina ranged between 10-12%. The samples were then stored at –4 °C before analysis.

Preparation of standard solutions for calibration

0.5 ppb OTA solution: 100 µL of OTA solution (10 ppb) were transferred into a glass vial via a pipette and was diluted using 1900 µL mixture of toluene: acetic acid (1:99 /v /v) yielding a 2 mL-volume solution.

5 ppb OTA solution: One hundred µL of OTA solution (0.5 ppb) were transferred into a volumetric flask with a 10 ml capacity. The solution was then steamed using nitrogen until it dried, after which it was dissolved in 10 ml mixture of a water: methanol (3/7v /v). The mixture was filtered using a 0.2 µm filter. The filtrate is stored at 4 °C to be used in the preparation of calibration solutions as shown in Table (1).

Table (1). Preparation of standard solutions for calibration

HPLC	Volume(in ul) of calibration solutions (5ppb each)	Volume (in µl) of (Water + methanol)	Concentrations (ppb)
1	25	4975	0.025
2	100	4900	0.11
4	250	4750	0.25
5	500	4500	0.5

OTA Extraction

Ten g were taken from each samples using a precision balance and put in 250 ml flasks. OTA was extracted by mixing well the sample with 200 ml of sodium hydrogen bicarbonate solution (1% w/v) on an Orbital Shafer for 30 minutes. The mixture was then filtered using filter no. 1 while pouring 10 ml of phosphate buffer saline (AOAC, 2004).

Washing the extracted specimen

Solid phase (SPE, RP18) (6 ml, Waters-Milford) columns were used for this process. The columns were first activated by passing through a 10 ml solution of phosphate regulator followed by 5 ml of distilled water. Once the activation was completed, a solution of each specimen's mixture in phosphate standard solution (10 ml /10 ml) was passed through the phase. The columns were then washed with 5 ml of distilled water and the samples were eluted with 3 ml methanol. The eluted solutions were then transferred into a conical amber tube with 4 ml capacity and were evaporated under nitrogen gas at 40oC.

Operating the high-performance liquid chromatography (HPLC)

The extract was dissolved in 500 ml of mobile phase and transferred in a 5 ml lidded flask via a 0.45 um needle, in order to prepare for the sample's injection in the HPLC. 20 ul each sample and standard solution were injected in the calibration curve. Using fluorescent detector, the study was performed setting the excitation wavelength at 330 nm, the emission wavelength at 477 nm, and the flow rate at 0.8 ml/min. The mobile phase consisted of a mixture of acetonitrile, methanol, phosphoric acid with a rate of (1.1.1 v/v/v), and separation column (C18: water-Milford) (3.9 × 150 mm 5 mm).

The validity of the results

The validity of the analysis method was verified using wheat samples free from OTA. Different concentrations of OTA, ranging between 1.0 and 0.025 ppb, were added to these wheat

samples. The lowest concentration detectable by chromatographic reached 0.025 ppb (see Table 2). The curve of standard solutions was prepared at five levels (0.025, 0.1, 0.25, 0.5, 1) ppb. This step was performed three times. The correlation coefficient reached 0.998. The average OTA recoveries from the samples was 94%, which demonstrates the effectiveness of the extraction method (see Table 3). OTA was identified by comparing retention time across test samples and standard solutions. The retention times were between 5 and 5.5 minutes. OTA was estimated by measuring the area under the curve at the time of OTA detention and comparing it with the calibration curve's area.

Table 2. Detection limits for spiked wheat samples of different concentrations of Ochratoxin A

Detection limits	Wheat samples (ppb)
0.025	0.025
0.09	0.1
0.24	0.25
0.47	0.5
0.99	1

Table 3. Recovery obtained from spiking of different levels of OTA in wheat samples.

%	OTA	
	Amount detected **(ppb)**	Amount added (ppb)
100	0.025	0.025
90	0.09	0.1
96	0.24	0.25
92	0.46	0.5
92	0.95	1

Results and Discussion

OTA presence in raw wheat grains and their derivatives:

This experiment was designed to detect the presence of OTA in raw wheat grains and their derivatives of semolina and bran collected from the General Company for Mills and Fodder in Tripoli factories. Table 4 shows the results of the analysis of all 96 samples taken during the milling process. It was found that 88 % (21/24) of the samples of unwashed raw wheat were contaminated. The minimum and maximum contamination concentrations ranged from 1.4 to 11.8 µg/kg and the average concentration was 5.9 µg/kg. The incidence of contamination decreased after cleaning the raw wheat samples as only 79% (19/24) of them were found contaminated. The concentration of OTA after cleaning varied 1.4-9.3 with an average of 5.3 µg/kg. For semolina, OTA is found in 12 samples out of 24 samples, and the minimum and maximum OTA concentrations ranged between 0.6 and 2.1 µg/kg, respectively. The average concentration was 1.2 µg/kg. Moreover, the study's results showed that the milling process led to a decrease in the level of OTA in semolina by 80 % as presented in Table 5. Opposite to semolina, the rate of contaminated bran samples rose to almost 83% (20/24), with OTA concentration rates ranging between 0.2 and 6.9 µg/kg. The average OTA concentration in bran samples was found to be 4.2 µg/kg. The results also showed that the majority of raw wheat samples (88%) contained OTA at a level slightly higher than the limit set in the Libyan Standard no231, data mostly reflect bad handling and storage practices as well lack of suitable control on such operations and in the same time it call for more tightened inspection in receiving of raw material in grain's mills under investigation. Meanwhile the average OTA concentration in all semolina samples (1.2 ug/kg) was below the Libyan National Centre for Standardization and Metrology (LNCSM) which permitted up to 3 µg/kg of OTA in this product. These results are, to a large extent, consistent with Duarte's (2010) findings, who found that 84% (57/70) of the raw hard wheat samples studied were contaminated with this compound,(at average concentrations 3.8 ppb), and that processing of wheat into semolina resulted in decrease in the concentration of OTA in this product by approximately 80% and are also generally consistent with Scudamore (2003) who stated that the initial cleaning processes remove between 25 to 35% of OTA concentration in wheat and milling process (purification and flour sifting) remove additional OTA percentages estimated between 35 and 75%. It is can be concluded from present study, that good production and manufacturing practices in handling and storage of such stable good should be followed and tightened inspection and examinations of incoming raw material to grain' s mills are strictly required.

Table 4. The proportion of contamination, minimum, maximum and average OTA concentration in hard wheat and outputs used in some mills in Tripoli.

Samples	Number of samples studied	Number of contaminated samples	Proportion of contamination (%)	OTA concentration (ppb)			
				minimum	Maximum	average	coefficient of variation
Crude Wheat	24	21	88	1.40	11.80	5.9 ±0.48	8.2
Wheat Cleaner	24	19	79	1.39	9. 30	5.3±0.32	6.1
Semolina	24	12	50	0.60	2. 10	1.2±0.02	1.2
Bran	24	20	84	0.70	6.90	4.2±0.34	8.1

Table 5.The effect of milling on OTA in semolina.

initial handling steps	OTA concentration (ppb)	% Reduction in the concentration of OTA	% Overall decline in the concentration of OTA	p- value
Receipt of raw wheat	5.9 ± 0.48	-----	-----	A
Cleaning	5.3 ± 0.32	10	10	B
Grinding and screening (Semolina)	1.2 ± 0.02	77.3	79.6	C

Conclusion

The finding of the present work implies that the managements of mills (concerned here) should give more care to the implementation of good practices in transportation, handling and storage of incoming wheat consignments as well as to tighten examinations and inspection procedures. It is seems likely that such manufacturing practices are not adequately followed in many of other national mills.

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