RESEARCH ARTICLE

Impact of Insect Densities *Tribolium Castaneum* on the Benzoquinone Secretions and Aflatoxins Levels in Wheat Flour During Storage Periods

Tarek A. El-Desouky[1,*], Samy S. Elbadawy[2], Hassan B.H. Hussain[2] and Nilly A. Hassan[2]

[1]*Food Toxicology and Contaminant, National Research Centre, Dokki, Giza, Egypt*
[2]*Plant Protection Research Institute, Agriculture Research Center, Dokki- Giza, Egypt*

Abstract:

Objectives:

The present study was prepared to investigate the impact of insect density, adult emergence of *Tribolium castaneum* on the secretion of Benzoquinones (BQs) consist of methyl-1,4-benzoquinone(MBQ) and Ethyl-1,4-Benzoquinone (EBQ), and accumulation of Aflatoxins (AFs) in wheat flour stored at different periods.

Methods:

Forty grams of wheat flour were put into small glass jars (8 cm diameter and 12 cm length). Then *T. castaneum* was put in each jars at rates of 10, 20 and 30 unsexed pairs of insect adult. The jars were covered with muslin cloth and the rubber band was fixed to prevent insects to escape. A glass jar without any insects served as the control. The jars lifted on bench in the laboratory for two, three and four months of storage under laboratory temperature conditions (with average $28 \pm 2C$ and 65 ± 5 R.H). The previous design was replicated three times. At the end of each storage period, the jars containing the flour were sieved thoroughly by 40 wire mesh size to separate the insects. The insects have been counted on the other hand wheat flour was prepared to determine MBQ, EBQ and AFs by HPLC methods.

Results:

The results indicated the levels of EBQ higher than MBQ in all infested samples at all insect densities (No. of insect pairs). The concentrations of MBQ in wheat flour released by ten adult pairs (10P) with the three storage periods two, three and four months were 10.42 ± 0.56, 22.38 ± 3.67, 27.06 ± 6.71 µg/g, respectively. These results increased with insect densities to (30p) were 39.67 ± 0.10, 63.58 ± 2.35 and 106.24 ± 7.4 µg/g after storage periods two, three and four months, respectively. In addition to the concentrations of EBQ with (10P) were 67.45 ± 3.64, 98.0 ± 6.1 204.66 ± 5.8 µg/g with storage periods two, three and four months, respectively. In case (30P) the levels of EBQ were 376.7 ± 0.87, 570.1 ± 2.11 and 1558.66 ± 10.88 (µg/g). The highest concentration of the BQs 1664.90 ± 11.43 (µg/g) released by *T. castaneum* achieved with the highest adult emergence (1021 insect adult) and the highest insect density (30p) at four months storage period. In general, AFs levels enhanced with a period of storage and insect densities.

Conclusion:

Levels of the BQs (MBQ and EBQ) increased with an increase of storage periods and insect densities. Therefore, the presence of this insect should be prevented in stored wheat flour reducing AFs contamination is possible by storage for short time and prohibit insects which causes an increase temperature of the flour and moisture, all of which promote production of AFs.

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* Address correspondence to this author at the Toxicology and Contaminant, National Research Centre, Dokki, Giza, Egypt; Tel: +201098635889; E-mail: eldesoukyt@yahoo.com

1. INTRODUCTION

The wheat flour is subjected to attack by many flour beetles, including the red flour beetle (*Tribolium castaneum*). The red flour beetle, *T. castaneum* is a polyphagous, cosmopolitan pest in flour mills and wherever cereal products and other dried foods were processed and/or stored. It is often the most common species in the pest complex attacking stored wheat. Mature flour beetles infect and/or contaminate the flour and give unpleasant odor and pinkish color [1], this negatively affects certain characteristics, such as viscous and elastic properties of the flour and create a disgusting taste. Some of the beetles have the ability to produce Benzoquinones (BQs) from specialized prothoracic and post abdominal glands. The chemical structures of the major components of these beetles secretions have been shown to consist of Methyl-1,4-Benzoquinone(MBQ) and Ethyl-1,4-Benzoquinone (EBQ), together with the carrier alkene 1-pentadecene. These compounds are hypothesized to function as external defense compounds, killing microbes and deterring predators, and their ability to evolve by natural selection depends on both selection and the genetic vs. environmental contribution to phenotypic variation [2, 3]. Many studies indicated that BQs produced or secreted by flour beetles may have toxic and carcinogenic effects on human and experimental animals [4 - 6]. Infestation of grains and stored products by insects promote growth of fungi including those that produce mycotoxins such as Aflatoxins (AFs) and results in contamination of commodities with insect bodies and waste products *etc*. Some of which are toxic, repulsive or allergenic [7]. AFs are group of secondary metabolites and highly carcinogenic produces by *A. flavus* and *A. parasiticus*. The four types major naturally produced known as Aflatoxin B_1 (AFB$_1$), Aflatoxin B_2 (AFB$_2$), Aflatoxin G_1 (AFG$_1$), and Aflatoxin G_2 (AFG$_2$). B and G refer to the blue and green fluorescent colors under UV light on Thin Layer Chromatography plates, while the subscript numbers related to their relative chromatographic mobility [8, 9]. Also (AFs) produced by *A. nomius*, *A. bombycis*, and *A. pseudotamarii* [10 - 13]. The insect also plays a significant role in the dissemination and proliferation of microorganisms including mycotoxigenic fungi in food commodities [14]. This work aims to study the effect of density of *T. castaneum* on the secretion of BQs and accumulation of AFs in wheat flour stored at different periods.

2. MATERIALS AND METHODS

2.1. Insect Cultures

The stock of insect used in this experiments (*T. castaneum*) was collected from stock culture maintained at stored grain and product pests Department, Plant Protection Research Institute, Agriculture Research Center, whereas they are reared at 28 ±2C and 65 ±5 R.H. on whole wheat flour for two months.

2.2. Experimental Procedures

Forty grams of wheat flour were put into small glass jars (8 cm diameter and 12 cm length). Then *T. castaneum* was put into each jars at rates of 10, 20 and 30 unsexed pairs of insect adult. The jars were covered with muslin cloth and the rubber band was fixed to prevent insects to escape. A glass jar without any insects served as the control

The jars were left on bench in the laboratory for two, three and four months of storage under laboratory temperature conditions (with average 28 ± 2C and 65 ± 5 R.H). The previous design was replicated three times. At the end of each storage period, the jars containing the flour were sieved thoroughly by 40 wire mesh size to separate the insects. The insects have been counted on the other hand, the wheat flour was prepared for determination of MBQ, EBQ and AFs by HPLC methods.

2.3. Extraction and Determination of BQs and AFs Using HPLC

BQs were extracted and determined according to Tomoskozi-Farkas and Daood method [15]. While AFs extracted and determined according to AOAC [16]

3. RESULTS AND DISCUSSION

3.1. Levels of BQs (MBQ and EBQ) in Wheat Flour Samples

Data represented in Table **1** showed the levels of MBQ and EBQ in samples wheat flour infested by *T. castaneum* at different storage period. The levels of MBQ during four month were 59.86 ± 10.94, 123.52 ± 11.57 and 209.49 ± 9.87

with 10P, 20P and 30P, respectively. While the level of EBQ in samples were 370.11 ± 15.6, 1498.25 ± 12.5 and 2505.46 ± 13.88 with 10P, 20P and 30P, respectively. The results indicated that the levels of EBQ higher than MBQ in all infested samples at all insect densities (No. of insect pairs) and storage periods.

Table 1. Levels of BQs in wheat flour samples infested by *T. castaneum* at different storage periods.

Storage Periods	Concentrations of BQs (µg/g)*						
		MBQ			EBQ		
	Control	10P	20P	30P	10P	20P	30P
Two Months		10.42±0.56	28.56±3.7	39.67±.1.1	67.45±3.64	199.5±2.6	376.7±0.87
Three Months		22.38±3.67	40.26±4.26	63.58±2.35	98.0±6.1	355.5±3.77	570.1±2.11
Four Months	ND	27.06±6.7	54.7±3.6	106.24±7.4	204.66±5.85	943.2±6.2	1558.66±10.88
Total during storage		59.86±10.94	123.52±11.57	209.49±9.87	370.11±15.6	1498.25±12.5	2505.46±13.88
BQs (µg/insect)		2.99	6.2	10.47	18.51	74.9	125.3

*mean±SD ND= Not detected; P= Pair of insect

These results agree with [17] found that the Quinone consists of 80 to 90% ethylquinone; 10 to 20 methyl quinone with a trace of other components. As well as Markarian *et al* [18] obtained 37% MBQ and 63% EHQ in the secretion of *T. castaneum*. These odorous BQs from *Tribolium* spp. have been well documented previously [19, 20]. Hodges *et al.* [21] did not found significant accumulations of quinones from beetles in dehusked rice, but contaminated wheat flour showed a more serious problem and the same two benzoquinone derivatives were reported and considered to be the secretions of *T. castaneum*.

3.2. Relationship between Insect Density of *T. Castaneum* and Levels of BQs Secretion

Data presented in Table **1** show increased levels of BQs (MBQ and EBQ) with an increase of the *T. castaneum* insect densities. The concentrations of MBQ in wheat flour released by ten adult pairs (10P) with the three storage periods two, three and four months were 10.42 ± 0.56, 22.38 ± 3.67, 27.06 ± 6.71µg/g, respectively. The result indicated that concentrations of MBQ increased with insect densities in case 30 adult pairs (30p) were 39.67 ± 0.10, 63.58 ± 2.35 and 106.24 ± 7.4 (µg/g) after storage periods two, three and four months, respectively. In addition to the concentrations of EBQ with (10P) were 67.45 ± 3.64, 98.0 ± 6.1 204.66 ± 5.8 µg/g with storage periods two, three and four months, respectively. In case (30P) the levels of EBQ were 376.7±0.87, 570.1±2.11 and 1558.66±10.88 (µg/g) at two, three and four months, respectively. In general, the total secretion rate of the two benzoquinones was increased with increasing of No. of pairs (insect density) of *T. castaneum* at the three storage periods. Senthilkumar *et al.* [22] found that the amount of volatiles produced by *T. castaneum* adults in wheat flour samples were a direct relationship which increased with an increase in insect density. The concentration of MBQ, EBQ and 1 tridecene released by ten adult insect were: 8.5, 9.1 and 10.6 µg/100 µl compared to 7, 8 and 4.2 µg/100µl for five adult insects after 72h storage period.

Results shown in Table **1** appeared that the concentrations of MBQ per insect were 2.99, 6.2 and 10.47 (µg/ insect) with 10P, 20P and 30P, respectively. In the same condition the concentrations of EBQ were 18.51, 74.9 and 125.25 µg/insect with the three insect densities (10p, 20p and 30p, respectively). These results agree with Unruh *et al.* [23] who reported that, in *T. castaneum* samples of newly eclosed adults, levels of MBQ and EBQ were very low, ≥ 0.1 and ≥ 0.3 µg per insect, respectively. The quinones increased with over time approximately to 40 - 50 days posteclosion. Where, the average total concentration of all quinones at 40 days posteclosion was 45 µg /insect, and the levels were as follows 18µg MBQ and 27 EBQ per insect. On the other hand, after 80 days, the amount of quinone was not significantly different from the overall total for a day with 40 beetles. Accumulation of quinones in flour wheat was probably due to an extended survival of the presence of the beetles in flour. However, the level of quinones changes from 9.2 to 472µg/g according on the species of flour beetles and storage periods [24, 25].

3.3. Effect of *T. Castaneum* Adult Emergence on BQs (Sum both of MBQ and EBQ) Secretion

Data illustrated in Table **2** showed that the secretion of BQs by *T. castaneum* adults increased with an increase in adult emergence with the three insect densities and storage periods. The highest concentration of the BQs 1664.90±11.43 (µg/g) released by *T. castaneum* achieved with the highest adult emergency (1021 insect adult) and the highest insect density (30p) at four months storage period, while, the lowest concentration level 77.87 ± 4.21 (µg/g) was recorded with the lowest insect density (10p) and adult emergency (550 adult) at the two months storage period. Also, in Table **2**, the other concentration values of the BQs were increased with the increasing of adult emergence with the

analogous insect densities and storage periods. These results are in agreement with Mondal [26] they showed that; levels of the quinones were low in newly emerged adults (< 20 µg per insect). While with age until 20 to 30 days after adult emergence the level of benzoquinones were increased. The levels were maintained for a long time after that time. In addition, females have higher levels of BQs than males of the same age.

Table 2. Effect of emerged adults on BQs and accumulation of total AFs in infested wheat flour by *T. castaneum* at different storage periods.

Insect Density	Storage Periods								
	2 Months			3 Months			4 Months		
	A.C.No of insect	BQs (µg/g)	AFs (µg/kg)	A.C.No of insect	BQs (µg/g)	AFs (µg/kg)	A.C.No of insect	BQs (µg/g)	AFs (µg/kg)
Control	0.0	ND	ND	0.0	ND	0.217±0.03	0.0	ND	0.75±0.09
10p	550.0±2.97	77.87±4.21	1.29±0.07	519.3±3.6	120.38±1.97	4.5±0.74	629.0±12.3	231.72±4.54	4.85±1..2
20p	663.0±8.63	228.1±2.9	3.31±0.43	918.4±4.74	385.68±4.09	3.54±0.37	958.33±6.29	997.9±6.55	4.4±0.3
30p	761.0±1.41	416.37±5.2	4.05±0.01	998.3±3.63	633.7±2.34	5.14±0.19	1021.0±7.01	1664.9±11.43	5.38±0.37

A.C.No=Adults cumulative number= Emerged adults; ND= Not detected

There are many interpretations to explain the low secretions of quinones from flour beetles with newly emerged *Tribolium* adults and then increased with increase of storage period time. Wirtz *et al.* [27] proposition that shortage of defensive secretion in newly emerged *Tribolium* adults reflects the need for an adequate barrier for self-protection. Only after that time does a rapid build-up of BQs occurs in adults. In another study, Mondal [26] did not show the detection secretion of the quinones in larvae or in prepupae, but quinones may be detected in some very late pupae and after one hour in adults emergence. There are many ways for quinones to enter into the flour, a great number of deaths of insects in a culture both in the laboratory and in the storehouse may lead to the extent of quinones in the flour medium [28]. Emptying of quinones is under some conditions such as crowding, excitement [1], agitation of the beetles [28] and partial narcosis [29]. The *Tribolium* spp. beetles outcome in the wheat flour becomes contaminated with quinone secretions thus, these quinone secretions may reduce the population of flour beetles. Fortunately, quinone secretion is highly toxic to the flour beetles themselves. In addition, Quinone secretion is able to producing different abnormalities in flour beetle populations; the effect varies with the developmental stages of the insects. Quinones cause reduced fecundity and fertility to *Tribolium*, therefore lessening of the reproductive rate [30]. On other hand, Happ [31] showed that tenebrionids are somewhat protected from their own secretions, within *Tribolium* species, this self-protection is evident in the partitioning of the secretion away from cells, first in the cuticle-lined organelles where the secretion is produced.

Previous studies indicate that BQs produced by flour beetles infesting the flour or grains may have a toxic effect on humans and animals was direct or indirect. Quinones can make infested flour unsuitable for human consumption and, flour may reach the point where it becomes toxic. Quinones are acutely toxic and allergenic as well as carcinogenic to human beings [5, 32, 33].

El-Mofty *et al.* [4, 6, 34] reported that, baking temperature did not reduce the carcinogenic effect of biscuits made from flour infested with *T. castaneum* beetles but the mutagenic effects on mice remains unclear after being contaminated flour had been cooked and consumed, and *Tribolium* spp. are the only storage pests producing carcinogenic and teratogenic contaminants. These compounds give an unpleasant smell to stored food and may be responsible for liver and spleen tumors in small vertebrates as well as bread prepared made from flour infested by *Tribolium* spp had a bad taste [35].

3.4. Effect of Storage Periods and Insect Density of *T. Castaneum* on AFs Levels.

Data illustrated in Table **3** showed that, there are relationships between storage periods and insect densities (10p, 20p and 30p) with foundation of AFs. We found significant relationship between the levels of AFG_1, AFB_1, AFG_2 and AFB_2 and total AFs with the insect densities and storage periods. The results revealed that generally, AFs levels increased with the period of storage and insect densities. Four-month stored infested flour sample had the highest total concentration of Aflatoxin (AFG_1, AFB_1, AFG_2 and AFB_2) with 4.85, 4.40 and 5.38 µg/kg, respectively with the insect densities (10p, 20p and 30p). Aflatoxin concentration levels of the two months storage period were 1.29±0.7, 3.31±0.43 and 4.06±0.2 µg/kg, respectively, while, total AFs of the three months storage period were 4.5±0.74, 3.54±0.37 and 5.14±0.2 µg/kg, respectively. However, AFG_1 and AFB_2 more had in concentrations than AFB_1 and AFG_2 in the stored

flour samples. Furthermore, total AFs increased with increasing of insect density with the exception that insect density 20p was decreasing at the three and four storage month's periods. These results confirmed by Jonathan, *et al*. [36] who reported that, aflatoxin levels increased with the period of storage. Eighteen-month stored flour sample had the highest concentration of AFB_1, AFB_2, AFG_1 and AFG_2 with 0.0033 µg/kg, 0.0085 µg/kg, 0.0080 µg/kg and 0.0065 µg/kg, respectively and generally, AFB_2 and G_1 had more concentrations than AFG_1 and G_2 in the stored samples.

Table 3. Concentration of AFs in infested wheat flour by T. *castaneum* with different storage periods.

AFs (µg/kg)	Control	2 Months			3 Months				4 Months			
		10p	20p	30p	control	10p	20p	30p	control	10p	20p	30p
AFG_1	ND	ND	0.963±0.13	3.7±0.01	0.217±0.03	3.78±.62	0.88±.1	1.74±0.06	0.33±0.04	3.88±0.96	0.85±0.06	1.72±0.12
AFB_1		1.29±0.07	0.169±0.02	ND	ND	ND	0.58±.06	0.85±.04	0.18±0.02	0.23±0.06	1.21±0.08	0.84±0.07
AFG_2		ND	ND	0.36±0.01	ND	0.72±.12	ND	ND	0.25±0.03	0.74±0.18	ND	ND
AFB_2		ND	2.18±0.28	ND	ND	ND	2.08±0.22	2.57±0.1	ND	ND	2.34±0.15	2.77±0.19
Total AFs		1.29±0.07	3.31±0.43	4.05±0.01	0.217±0.03	4.5±0.74	3.54±0.37	5.14±0.19	0.75±0.09	4.85±1.2	4.4±0.3	5.38±0.38

ND= Not detected

Among fungal toxins, which have been noticed in several studies, AFs are a large group of mycotoxins which are produced by some species of *Aspergillus* spp on foods such as cereals including (wheat, corn and barley) as well as legume, nuts and feed. These species have a worldwide prevalence. Diseases in animals and human by AFs called aflatoxicosis. Acute aflatoxicosis, associated with extremely high doses of AFs, is characterized by hemorrhage, acute liver damage, edema, and death in humans. Conditions increasing the likelihood of acute aflatoxicosis in humans include limited availability of food, environmental conditions that favor fungal development in crops and commodities, and lack of regulatory systems for AFs monitoring and control. On the other hand, these fungal contaminations not only pose a serious health risk to consumers but also diminish the nutritional value and economic benefits of the food. Wheat contamination with fungi and AFs has been noted in several studies, the presence of high levels of aflatoxins in stored foods may made it unacceptable for marketing, causing financial loss to the farmers or retailers [37, 38]. The maximum level of AFs cereal, wheat flour and cereal products according to European Union (EU) and Egyptian Standard (ES) were 4 and 2µg/kg. Accordingly, five samples exceeded the maximum set in the EU and ES. Cereals and other crops are exposed to fungal attack in the field (pre-harvest) or during storage and this would result in the production of AFs. Also the climatic and storage conditions practices also play important role in fungal attack and mycotoxins production. Due to improper storage conditions in urban areas of Egypt. It also has been reported that increased AFs formation was registered by heavy rains during the storage, by delayed storage and high moisture contents [39, 40]. Tirado *et al*. [41] reported that the AFs are expected to become more prevalent with climate change in countries with temperate climate which has not with this problem before.

CONCLUSION

Stored wheat flour should be inspected frequently at a regular interval to detect whether they have been infested by flour beetles through the presence of quinones in flour by HPLC analysis. Consequently, these benzoquinone compounds which were produced by adults might be used as biomarkers for detection of *T. castaneum* in flour or grain. The results of this study indicate that the levels of the BQs (MBQ and EBQ) increased with an increase of storage periods and insect densities. Therefore, the presence of this insect should be prevented in stored wheat flour. In this study, reducing AFs contamination is possible by reducing wheat flour storage time, humidity and insects, causing an increase in temperature of the flour and moisture, all of which promote the production of AFs.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Declared none.

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