Investigation for Pu-Erh Tea Contamination Caused by Mycotoxins in a Tea Market in Guangzhou

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Abstract: *Objective*: The purpose of the present study is to provide raw data for the development of guidelines for tea production and management, as well as relevant health standards. To investigate the mycotoxin contamination in the wet stored Pu-erh tea in a tea market in Guangzhou, we measured the concentrations of aflatoxin B_1 (AFB_1), fumonisin B_1 (FB_1), deoxynivalenol (DON), and T-2 toxin in 70 tea samples.

Methods: 70 samples of wet stored Pu-erh tea were randomly chosen in the market. Following crushing, brewing, and filtration of the samples, the contaminations of FB_1, DON, or T-2 toxin were assayed by ELISA detection kits, and the contamination of AFB_1 was measured by the IAC-HPLC method.

Results: We found that all tea samples were safe regarding FB_1 and T-2 toxin (safety limit, 1 mg/kg and 0.1×10^{-3} mg/kg, respectively). However, 8 out of 70 samples displayed higher AFB_1 concentrations compared to the safety limit (5×10^{-3} mg/kg). Surprisingly, 63 out of 70 samples have exceeded the safety limit for DON (1 mg/kg).

Conclusion: Our survey was the first time to find AFB_1 and DON contaminations in the wet stored Pu-erh tea in this tea market. Although the FB_1 and T-2 toxin in these tea samples has not yet exceeded the safety limits, they were still detectable, which should cause more concern.

Keywords: Pu-erh tea, Aflatoxin, fumonisin, deoxynivalenol, T-2 toxin.

INTRODUCTION

Mycotoxins are biological toxins that are produced by fungi (for example, aspergillus, penicillium, and fusarium). To date, more than 200 types of mycotoxins have been discovered, which were classified into four major categories: aspergillus toxins (e.g. aflatoxin, brown song adriamycin), penicillium toxins (e.g. patulin, orange green toxins), fusarium toxins (e.g. fusarium enol, zearalenone), and others (such as spores poison). Mycotoxins are harm to human health through contaminating grains and animal food such as milk, meat, and eggs. Mycotoxins can take effect through a variety of mechanisms, for instance, suppression of the human immune system and hematopoietic system, neuroendocrine disorders, and liver and kidney damage. Mycotoxins can also cause endemic diseases. More seriously, some mycotoxins have been proved to be able to induce cancer, deformity, and gene mutagenesis.

China is a major country that is engaged in tea production, export and consumption. In China, mildew has always been detected in the wet stored Pu-erh tea, probably because the environment for the tea production is suitable for fugi growth. Hence, we investigated the contamination caused by AFB_1, DON, FB_1, and T-2 toxin in the samples of wet stored Pu-erh tea, to provide raw data for the improvement of Pu-erh tea production, and development of bylaws for human health safety.

1. MATERIALS AND METHODS

1.1. Major Reagents

The AFB_1 standard was kindly provided by AXXORA (Exeter, UK). The detection kits for FB_1, DON, and T-2 toxin were kindly provided by Century Trading Co., Ltd. (Beijing, CHINA). The detection kit consists of: micro-plate containing goat anti-rabbit IgG antibodies, negative control, toxin standard, HRP marker for toxins, rabbit anti-toxin antibody, substrate, and stop solution.

1.2. Samples

1.2.1. Sample Collection

70 samples were randomly chosen on August 7th, 2011, from the wet stored Pu-erh tea in a tea market in Guangzhou, P.R. China.

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1.2.2. Sample Preparation

The samples were prepared according to the manual. Briefly, the samples were crushed, followed by filtration using a 20-mesh sieve. The samples were then resuspended in 70% methanol or distilled water, shaken for 3 min, and incubated under room temperature for 5 min. The supernatants were collected and filtered using a 0.45 μm disposable filter.

1.3. Methods

For each sample, the preparation and toxin detection procedures were repeated three times. The weighted average was used as the effective concentration of aflatoxin.

1.3.1. AFB_1 Test

The IAC-HPLC (Immunoaffinity chromatography purification-High Performance Liquid Chromatography) method was conducted according to the national standard [1]. The major steps are as follows: After preparation as described in 1.2.2 and dilution, the samples were purified by affinity chromatography with AFB_1 specific antibodies and PBST, and eluted by methanol. The AFB_1 concentrations in the eluted sample solutions were determined by measuring the iodine derivative fluorescence with the fluorescence reader in the high performance liquid chromatography column.

1.3.2. FB_1, DON, and T-2 Toxin Tests

ELISA tests were conducted according to the manual. Briefly, after preparation as described in 1.2.2 and dilution, 50 μl sample solution was added to each well of the ELISA plate and mixed with 50 μl anti-toxin antibodies and 50 μl HRP solution. In the meanwhile, the toxin stand group and the control group were also established. Following shaking at room temperature for 10 min, the plate was rinsed with cleaning fluid five times, added with 100 μl substrate, and shaken at room temperature for 5 min. The ELISA reaction was terminated, and the absorbance at 450 nm (OD_{450}) was measured. The standard curve was constructed by plotting the mean relative absorbance (%) obtained from each reference standard against its concentration in ng/mL on a logarithmic curve. And use the mean relative absorbance values for each sample to determine the corresponding concentration of the tested sample in ng/mL from the standard curve.

$$\text{Relative absorbance (\%)} = \frac{\text{absorbance standard (or sample)}}{\text{absorbance zero standard}} \times 100$$

2. RESULTS

When detecting AFB_1, quantification was performed using external calibration with mixed standards containing 0.10mg/kg of AFB_1 with the IAC-HPLC (Immunoaffinity chromatography purification - High Performance Liquid Chromatography) method. When detecting FB_1, DON, and T-2 toxin, the calibration curves are linear over the whole range correspondingly.

2.1. AFB_1 Concentration

The IAC-HPLC analysis showed that AFB_1 could be detected in all samples. Among the 70 samples, 8 (11.43%) displayed higher AFB_1 concentration than the national safety limit (5×10^{-3} mg/kg) (note: here the kg means per kilo gram dry tea, and the following are the same). And 3 of these 8 samples displayed a concentration even higher than 7.5×10^{-3} mg/kg.

2.2. FB_1 Concentration

As shown in Figure **1**, the standard curve was constructed by plotting the mean relative absorbance, the regression equation is as follows: "y = -0.1302Ln(x) + 0.6929" and the correlation coefficient R^2 = 0.9947. The results of the ELISA reactions are shown in Table **2**. Compared to the safety limit of 1 mg/kg, which was determined by the former Swedish government on corn and related products [2], all samples displayed a low concentration of FB_1.

2.3. DON Concentration

As shown in Figure **2**, the standard curve was constructed by plotting the mean relative absorbance, the regression equation is as follows: "y = -0.1549Ln(x) + 0.8488" and the correlation coefficient R^2 = 0.9948. The results of the ELISA reactions are shown in Table **3**. Compared to the safety limit of 1 mg/kg, which was determined by the index of "GB2761-2005 limits of mycotoxins in foods", 63 out of 70 (90%) samples exhibited a higher concentration of DON. Moreover, 16 samples exhibited a concentration even higher than 2.5 mg/kg, as shown in Table **3**.

2.4. T-2 Toxin Concentration

As shown in Figure **3**, the standard curve was constructed by plotting the mean relative absorbance, the regression equation is as follows: "y = -0.1797Ln(x) + 0.9035" and the correlation coefficient R^2 = 0.9994. The results of the ELISA reactions are shown in

Table 1: The AFB_1 Concentration of Pu-Erh Tea Samples (mg/kg)

No.	AFB_1 level ($\times10^{-3}$)	No.	AFB_1 level ($\times10^{-3}$)	No.	AFB_1 level ($\times10^{-3}$)
1	0.236±0.0215	25	1.254±0.1154	49	3.681±0.3578
2	0.975±0.0887	26	2.192±0.2085	50#	8.164±0.8055
3	1.592±0.1453	27#	5.465±0.5123	51	3.328±0.3215
4	0.768±0.0748	28	1.261±0.1123	52	4.053±0.3987
5#	5.294±0.5026	29	2.931±0.2845	53	3.574±0.3356
6	0.931±0.0897	30	1.227±0.1023	54	0.021±0.0012
7	1.425±0.1365	31	2.134±0.2013	55#	8.521±0.8243
8	0.975±0.0876	32	3.761±0.3645	56#	6.342±0.6124
9	2.495±0.2356	33	1.261±0.1124	57	2.381±0.2145
10	1.418±0.1245	34	4.342±0.4123	58	1.325±0.1235
11	2.787±0.2654	35	2.937±0.2856	59#	7.312±0.7256
12	2.955±0.2986	36	1.264±0.1125	60	2.322±0.2133
13	2.194±0.2153	37	2.965±0.2745	61	0.583±0.0125
14	2.119±0.2014	38	3.261±0.3121	62	2.137±0.2156
15	1.425±0.1325	39	1.124±0.1120	63	3.021±0.2589
16	2.145±0.2056	40#	5.654±0.5242	64	1.325±0.1325
17	3.326±0.3214	41	1.638±0.1542	65	0.853±0.0786
18	2.480±0.2315	42	4.418±0.4524	66	1.211±0.1322
19	0.931±0.0863	43	2.937±0.2856	67	2.104±0.1988
20	3.731±0.3545	44	3.266±0.3122	68	4.627±0.4536
21	2.937±0.2976	45	4.532±0.4215	69#	5.285±0.5121
22	2.143±0.2013	46	1.775±0.1566	70	0.315±0.0286
23	2.937±0.2856	47	4.342±0.4125		
24	2.367±0.2153	48	1.024±0.0987		

#Beyond the limit of national standard.

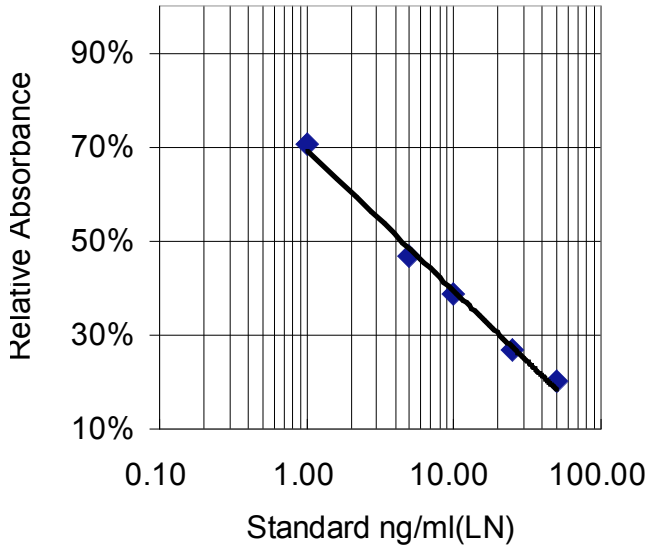

Figure 1: the standard curve of ELISA reactions for FB1.

Table 2: Levels of FB_1 (mg/kg)

No.	FB_1 level	No.	FB_1 level	No.	FB_1 level
1	0.267±0.0259	25	0.357±0.0347	49	0.128±0.0115
2	0.037±0.0032	26	0.222±0.0211	50	0.117±0.0102
3	0.236±0.0214	27	0.182±0.0168	51	0.107±0.0098
4	0.258±0.0235	28	0.186±0.0174	52	0.102±0.0097
5	0.427±0.0415	29	0.155±0.0152	53	0.102±0.0097
6	0.499±0.0452	30	0.199±0.0198	54	0.050±0.0046
7	0.225±0.0214	31	0.130±0.0121	55	0.066±0.0052
8	0.486±0.0423	32	0.334±0.0312	56	0.093±0.0089
9	0.332±0.0214	33	0.161±0.0154	57	0.090±0.0091
10	0.376±0.0265	34	0.157±0.0142	58	0.097±0.0098
11	0.257±0.0231	35	0.141±0.0132	59	0.042±0.0036
12	0.269±0.0254	36	0.124±0.0112	60	0.016±0.0015
13	0.228±0.0213	37	0.075±0.0065	61	0.037±0.0029
14	0.327±0.0312	38	0.193±0.0186	62	0.021±0.0013
15	0.372±0.0354	39	0.190±0.0186	63	0.068±0.0046
16	0.386±0.0356	40	0.169±0.0154	64	0.042±0.0038
17	0.372±0.0251	41	0.106±0.0981	65	0.134±0.0139
18	0.361±0.0361	42	0.194±0.0187	66	0.071±0.0065
19	0.185±0.0187	43	0.049±0.0036	67	0.065±0.0056
20	0.197±0.0195	44	0.056±0.0046	68	0.063±0.0058
21	0.156±0.0135	45	0.145±0.0132	69	0.030±0.0026
22	0.228±0.0209	46	0.105±0.0097	70	0.093±0.0078
23	0.325±0.0315	47	0.152±0.0146		
24	0.186±0.0184	48	0.195±0.0187		

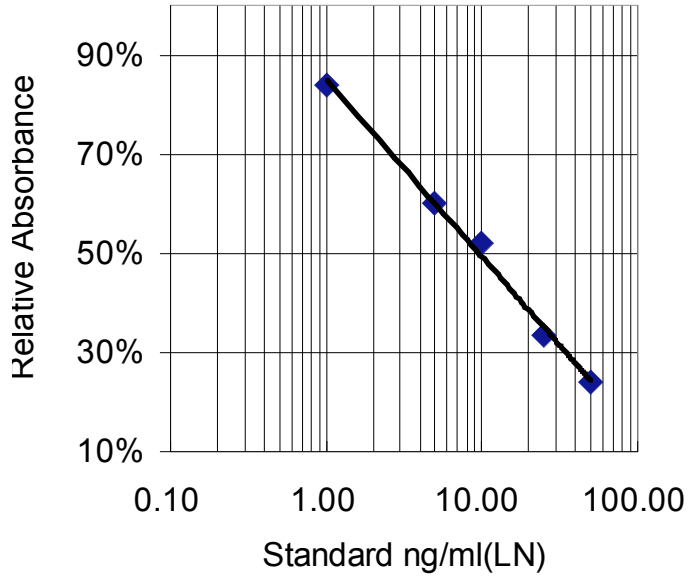

Figure 2: the standard curve of ELISA reactions for DON.

Table 3: Levels of DON (mg/kg)

No.	DON level	No.	DON level	No.	DON level
1	1.044±0.1002	25	1.663±0.1053	49#	2.777±0.2602
2	0.357±0.0356	26	1.585±0.1469	50#	2.563±0.0498
3	1.559±0.1456	27	1.132±0.1069	51#	2.868±0.2788
4	1.663±0.1589	28	2.255±0.2089	52	2.291±0.2156
5	2.328±0.2145	29	2.184±0.1098	53#	2.689±0.2586
6	1.690±0.1523	30	1.372±0.1126	54#	2.605±0.2486
7	1.169±0.1026	31	2.184±0.2019	55#	2.822±0.2787
8	1.921±0.1914	32	2.149±0.2014	56	1.717±0.1654
9	0.795±0.0056	33	2.184±0.2067	57#	2.777±0.2685
10	1.636±0.1546	34	1.745±0.1652	58	2.184±0.2015
11	0.890±0.0849	35	2.255±0.2106	59	1.745±0.1654
12	1.207±0.1023	36	2.366±0.2236	60	2.443±0.2154
13	0.980±0.0189	37	1.559±0.1456	61	2.366±0.2213
14	2.115±0.1985	38	0.758±0.0748	62#	2.647±0.2546
15	2.081±0.1956	39	1.510±0.1458	63#	2.914±0.2985
16	2.115±0.1985	40	1.463±0.1326	64#	2.733±0.2654
17	1.952±0.1869	41#	2.605±0.2512	65#	3.009±0.2989
18	2.081±0.1986	42	0.934±0.0921	66	2.328±0.2148
19	1.890±0.1758	43	2.366±0.2154	67#	3.107±0.3021
20	1.860±0.1769	44	1.860±0.1659	68#	2.733±0.2614
21	1.745±0.1598	45#	2.647±0.2521	69	1.585±0.1489
22	1.486±0.1326	46	1.860±0.1068	70	0.469±0.0457
23	2.219±0.2102	47	2.328±0.2145		
24#	2.522±0.2471	48	1.012±0.1006		

#Beyond the limit of national standard.

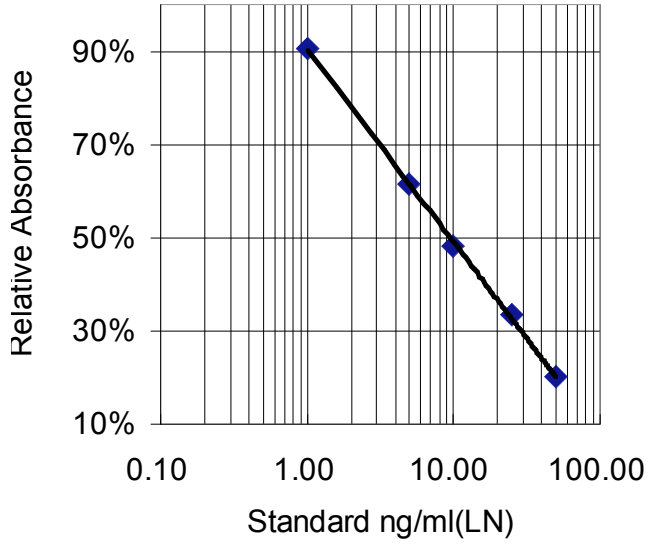

Figure 3: the standard curve of ELISA reactions for T-2 toxin.

Table 4: Levels of T-2 Toxin (mg/kg)

No.	Levels of T-2 toxin ($\times10^{-3}$)	No.	Levels of T-2 toxin ($\times10^{-3}$)	No.	Levels of T-2 toxin ($\times10^{-3}$)
1	47.742±4.2581	25	18.291±1.7892	49	13.042±1.2085
2	19.213±1.8722	26	9.828±0.9758	50	5.184±0.4987
3	10.135±1.0012	27	11.183±1.0078	51	9.950±0.9658
4	13.615±1.3025	28	8.479±0.8026	52	11.532±1.1025
5	12.415±1.2023	29	14.127±1.4256	53	11.675±1.1023
6	13.284±1.2156	30	8.744±0.8954	54	21.862±2.2356
7	7.732±0.6988	31	11.892±1.1026	55	17.628±1.7584
8	18.067±1.7589	32	26.617±2.5956	56	19.692±1.8659
9	14.658±1.3568	33	8.691±0.8546	57	23.828±2.3254
10	10.073±1.0012	34	10.135±1.0032	58	15.209±1.4985
11	13.366±1.3120	35	13.284±1.2536	59	14.390±1.3859
12	13.869±1.3186	36	9.017±0.8652	60	13.203±1.3210
13	13.784±1.3658	37	11.046±1.0089	61	16.374±1.5418
14	17.846±1.7586	38	11.819±1.1056	62	9.889±0.0954
15	34.462±3.2645	39	9.356±0.8978	63	19.095±1.8975
16	29.733±2.8596	40	16.885±1.5658	64	12.725±1.2658
17	18.746±1.5878	41	12.803±1.2145	65	18.067±1.8548
18	10.073±1.0025	42	13.869±1.3654	66	9.242±0.8569
19	14.214±1.3589	43	12.039±1.2103	67	8.962±0.8545
20	13.784±1.2956	44	18.067±1.8659	68	8.852±0.8269
21	13.203±1.2356	45	22.133±2.2105	69	9.242±0.8978
22	15.976±1.4987	46	18.746±1.8654	70	7.876±0.6859
23	15.878±1.4985	47	13.954±1.2654		
24	19.451±1.8956	48	23.975±2.3758		

Table **4**. Compared to the safety limit of 100 μg/kg (determined by the former USSR), each sample displayed a low concentration of T-2 toxin.

3. DISCUSSION

Aflatoxins have been confirmed to be strong mutagens and liver carcinogens for many species including the human [3]. Hence, in 1993, they were determined as Class I carcinogens by the World Health Organization (WHO) [4, 5]. Aflatoxins are polycyclic steroid compounds that are produced by aspergillus and their metabolites. To date, 17 isoforms of aflatoxins have been discovered, among which the most important are B_1, B_2, G_1, G_2, and M_1. Being most common in food, AFB_1 possesses the highest toxicity and carcinogenic activities, followed by: M_1> G_1> B_2> G_2. The toxicokinetic analysis of AFB_1 revealed that it can be easily absorbed by the gastrointestinal tract and peritoneum [3], thus the concentration of AFB_1 in plant products has been strictly limited by most countries. For instance, in China, the AFB_1 concentration in cereals (e.g. corn) and their related products is limited to no more than 5×10^{-3} mg/kg [6].

A new type of strong biological toxin produced by fusarium moniliforme, fumonisins, was first discovered in 1989. To date, seven isoforms of fumonisins have been found. Fumonisins take effect through their activities of ceramide inhibition and interference with the biosynthesis of nerve sheath ammonia alcohol lipids. In addition, FB_1 can also induce oxidative reactions in renal cells, which in turn may lead to damage to cells and DNA [7, 8]. It has been suggested that FB_1 can cause liver damage and cardiovascular dysfunction [9], which subsequently results in ELEM,

PPE [10], and – possibly - human esophageal cancer. Fumonisins exist largely in corn and its products. In Korea and South Africa, the concentration of FB1 in corn and its products is limited to no more than 7.9 mg/kg. In Sweden, FB_1 in food is limited to no more than 1 mg/kg [2].

DON is a secondary metabolite of F. Fusarium, and always coexists with other fungal toxins. DON is highly toxic, and its oral LD_{50} for rats is 7.3 mg/kg. The DON contamination in food can cause acute poisoning symptoms, including anorexia, vomiting, diarrhea, fever, unstable standing, and delayed responding. It has been suggested that DON can directly induce DNA damage and cell apoptosis [11]. In China, according to the national standard "GB2761-2005: limits of mycotoxins in foods", DON is limited to no more than 1 mg/kg in wheat and corn.

T-2 toxin and its metabolite HT-2 are one type of highly thermal stable toxin that is produced by fusarium. T-2 and HT-2 toxins mainly cause diseases in the immune system and the blood, such as alimentary toxic aleukia (ATA) [12]. In addition, T-2 toxin is famous for its possible role – though not yet verified – in the induction of the endemic Kashin-Beck disease. In 2001, the European Food Science Committee determined the maximal capacity for short-term T-2/HT-2 co-intake to be 0.06×10^{-3} mg/kg/day [13]. Also, the former USSR had ever determined that the T-2 toxin concentration in cereals should not exceed 0.1 mg/kg.

Since fungi opt for living their entire lives within plants, their metabolites and toxic products can easily contaminate plant products and subsequently cause intoxication. Pu-erh tea, especially the wet stored one, is likely to be contaminated by fungi and their toxins, because of its parasite-friendly processes of production, storage, and transport. To investigate the mycotoxin contamination in the wet stored Pu-erh tea, we measured the concentrations of four types of mycotoxins in 70 Pu-erh tea samples that were randomly chosen from 12 stores in a tea market in Guangzhou. Because people directly brew tea for drinking, our measurements were based upon the soaking solution of the tea samples. The purpose of this study is to provide raw data to further support the health management of Pu-erh tea.

Our results showed that both AFB_1 and DON induced contamination in different numbers of tea samples, and in some cases the toxin concentration even exceeded the safety limit by twofold. Among the contaminated samples, the contamination by these two types of toxins did not display any statistically significant correlation. This, as it is, the actual phenomenon because the two mycotoxins are produced by different molds under different conditions. Till today, the safety limit of AFB_1 in tea has not been determined. However, as AFB_1 is a strong carcinogen, more effort should be put into the prevention and elimination of contamination caused by AFB_1. Also, the same actions should be taken for DON due to its strong toxicity. Furthermore, as for FB_1, the current national and international research has been focused on corn and its products, but not on other plants such as tea. Hence, in this study, we investigated the FB_1 contamination in Pu-erh tea. According to the FB_1 safety limits determined by several countries, FB_1 did not cause any significant contamination in our tea samples. Given the potential toxicity of FB_1, continuous efforts should be made regarding the prevention of FB_1 contamination in tea. Finally, our results about T-2 toxin revealed that all the tea samples displayed a concentration that far below the safety limit of 0.1×10^{-3} mg/kg, indicating that Pu-erh tea is not a good host for fusarium, which can produce T-2 toxin.

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