The Effect of Dipping Pretreatment on Ochratoxin A Accumulation in Sultanas and Currants

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Abstract The dipping pretreatment on the occurrence of ochratoxin A (OTA) in sultanas and currants was investigated. Grape samples were divided into two groups before a drying process. One group was dipped with potassium carbonate-ethyl oleate, and the other group was left without treatment (the control). OTA were detected using solid-phase extraction clean-up and a high-performance liquid chromatography fluorescence detector. Results showed that OTA content was below the limit of detection in the fresh grapes of both varieties. OTA were not detected in dried vine fruits before storage. However, a real naturally environmental storage revealed that the dipping pretreated samples were contaminated with OTA earlier and to a greater extent than the control. The OTA concentration in the treated and control samples after one-year storage was 0.22 and 0.19 $\mu\text{g/kg}$ for sultanas and 0.34 and 0.21 $\mu\text{g/kg}$ for currants, respectively. These results indicated that dipping pretreatment might increase the toxin contamination and safety risk.

Keywords: Ochratoxin A, dipping pretreatment, Thompson seedless, Flame seedless, storage

Introduction

Dried vine grape is an important grape-derived product. It is one of the favorite dried fruits throughout the world. This dried fruit is fat and cholesterol free and contains essential vitamins, minerals, and 70% fructose. Besides these nutrients, raisins possess polyphenol and flavonoid antioxidant compounds that contribute to good human health (1,2). Dried vine fruits are normally classified into three groups according to grape variety: raisins (dried white grapes), sultanas (dried white grapes from seedless varieties), and currants (dried red seedless grapes).

Ochratoxin A (OTA) is a mycotoxin with nephrotoxic, immunotoxicity, and teratogenic properties and has been recognized as a possible human carcinogen according to the International Agency for Research of Cancer (3). OTA has been detected in many foodstuffs and beverages such as beer, grape juices, musts and wines, grapes, and dried vine fruits (4–6). It has been found that dried vine fruits contained the highest OTA content among grapes and their derivatives; in some surveys, the OTA contamination of dried vine fruits reached 50–70 $\mu\text{g/kg}$ (5,7). The occurrence of OTA in dried vine fruits has been studied in many countries such as Argentina, Greek, Canada, and China (8–11). The maximum level of OTA allowed in dried vine fruits in Europe is 10 $\mu\text{g/kg}$ (12). Moreover, *Aspergillus niger* aggregate and *Aspergillus carbonarius* have been blamed as the OTA producers in grape and grape-derivatives (13–15). Astoreca

et al. (16) reported that, throughout the grape drying process, the biodiversity index showed a decreasing trend because the water activity (A_w) of grape was decreasing and the sugar level was increasing. *A. carbonarius* and *A. niger* aggregate can grow under strict conditions.

Turpan, located in Sinkiang Uyghur Autonomous Region, is a major raisin-producing region in China. In this region, grapes are always dehydrated in cooling chambers called Chunche. Each chamber has a roof and walls with a large number of holes, which allow hot and dry winds to pass through and thus accelerate the drying process through evaporation. Grapes dried by this technique provide dried fruits that are bright in color, while the sun-dried fruits result in a dark color. Before drying, grapes are always pretreated with an alkaline-oleate solution referred to locally as “dry promoter.” In previous studies, various chemical-dipping pretreatments had been used, including potassium carbonate-ethyl oleate, sodium hydroxide-ethyl oleate, and sodium hydroxide-potassium carbonate-ethyl oleate (17,18). The dipping pretreatment not only reduces the drying time, increasing the economic benefit, but also improves the color, flavor, and nutritional quality of dried vine fruit (17,19).

The waxy components are on the surface of grapes, offering grapes a high resistance to moisture transfer and protection against fungal infection. However, it has been found that ethyl oleate dissolves the wax layer on the surface of grapes (19). The thickness of grape skin can affect OTA contamination (20). Moreover, the

thickness and structure of the grape wax layer and cuticle can affect the fungal colonization (21). Therefore, the dipping treatment can cause the loss of wax on the surface of grapes, inducing a higher possibility of fungal infection. However, little attention has been paid to the effect of dipping pretreatment on the OTA accumulation in the grape dehydration process and the dried vine fruits storage period. In our previous study, we found that currants have a higher risk of OTA contamination than sultanas, so two varieties of dried vine fruit were tested in this study (11). Thus, the purposes of this work were to evaluate the effect of dipping pretreatment on OTA content in the final dehydrated grapes, determine its changes during a storage period, and explain the potential reasons for such a change in OTA content.

Materials and Methods

Samples Fully ripened *Vitis vinifera* Thompson seedless grapes and *Vitis vinifera* Flame seedless grapes were harvested from the Sinkiang test field of the Grape and Melon Research Center (Shanshan, Turpan, Sinkiang, China) in 2012 (Table 1). Before the drying process, the dipping pretreatment was performed. Dipping pretreatment solution (named cu-4) was provided by Sinkiang HuiPu Garden Technology Company (Urumchi, Sinkiang, China). Its primary ingredients were 3.5% potassium carbonate and ethyl oleate. Both pretreated and control group (without dipping application) grapes were dried in a Chunche chamber (Shanshan, Turpan, Sinkiang, China). The final desired moisture content was 16–18% (w/w).

Both Thomson and Flame seedless grapes were picked on August 31, 2012 and then dehydrated in Chunche chambers. The grapes with dipping pretreatment were dried for 27 days, while the control group became dried fruits in almost 38 days.

After dehydration, all the Thompson seedless sultanas and Flame seedless currants were stored in bulk in a natural environment for up to 12 months. Located meteorological data were collected from the China Meteorological Data Sharing Service System. The samples were collected every month and their OTA concentration determined. Each variety and each group had a total of 10 bins, and each bin contained about 25 kg of samples. Sampling was conducted from the top, middle, and bottom of each bin, and each portion was approximately 100 g. The number of incremental samples was 30, and the total amount was about 3,000 g. The collected samples were homogenized, and the resultant homogenate (200 g) was used for analysis (22).

Reagents and materials OTA crystal power was obtained from Sigma-Aldrich (St. Louis, MO, USA). The stock solution was prepared by dissolving the OTA standard in methanol to a concentration of 32 mg/mL. The working solution was prepared by diluting the stock solution with methanol to the concentration of 50 µg/L. All the standard solutions were stored at 4°C in the dark. Methanol,

acetonitrile, and acetic acid were high-performance liquid chromatography (HPLC)-grade and purchased from Merck (Darmstadt, Germany). Analytical grade sodium hydrogen carbonate and hydrochloric acid were purchased from Beijing Chemical Works (Beijing, China). Ultrapure grade water was obtained through a Milli-Q gradient system at 18 mΩ (Molsheim, France). C18 solid-phase extraction (SPE) columns (3 mL, 500 mg) were obtained from Waters (Milford, MA, USA).

Extraction and purification of OTA The extraction and purification procedures used to obtain OTA from these dried vine fruit samples were the same as those used in our previous work (11). The dried fruit samples were soaked overnight in 0.8 times their weight in water and then homogenized in a blender (HR2860; Philips, Zhuhai, China). Forty-five grams of the sample was transferred to a 250 mL conical flask. The grape samples were slurried directly without soaking, and 45 g was weighed out for extraction. Then, the sample was extracted with 40 mL of 1.5% sodium bicarbonate and 140 mL of methanol, and then vortexed for 3 min using a high-speed blender (T18; IKA, Königswinter, Germany). The extracting solution was filtered and adjusted to pH 6–7 using 1 M HCl. The filtrate (10 mL) was diluted with 10 mL of Milli-Q water. C18 SPE columns were preconditioned using 2 mL of acetonitrile and 2 mL of water. Afterward, 20 mL of diluted solution and 10 mL of water were passed alternately through the C18 SPE column (2 mL extract and 1 mL water) at a flow rate of about 1 drop per second. OTA was eluted with 2 mL of methanol-acetic acid solution (99.5:0.5, v/v). The resulting eluent was dried under a nitrogen stream at 45°C and reconstituted with 500 µL of the HPLC mobile phase. Each sample was analyzed in triplicate.

HPLC analysis The HPLC method used was based on a previous method (11). The HPLC apparatus was equipped with a LC-20AT pump, a PF-20 Axs fluorescence detector, and a Lab Solutions/LCSolution software data analysis system (Shimadzu, Tokyo, Japan). The mobile phase consisted of (A) 1% glacial acetic acid (v/v) in water and (B) 1% glacial acetic acid (v/v) in acetonitrile. The separation was performed using a Phenomenex Prodigy ODS3 column (250×4.6 mm, 5 µm; Phenomenex, Torrance, CA, USA) with the isocratic elution of A:B=1:1 (v/v). The injection volume was 20 µL with a flow rate of 1.0 mL/min. The excitation and emission wavelengths of the fluorescence detector were 333 and 460 nm, respectively. Figure 1 shows the HPLC chromatograms of OTA in a standard solution, the control samples after one-year storage, and dipping pretreated samples after one-year storage.

The limit of detection (LOD) of this method was 0.07 µg/kg, while the limit of quantification was 0.16 µg/kg (11).

Statistics Statistical results were calculated using Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL, USA) version 16.0 for Windows. A student *t*-test was used to evaluate the significant

Fig. 1. (A) Chromatogram of an ochratoxin A standard solution (0.25 µg/L); (B) chromatogram of dipping pretreated sultanas after one year of storage (0.22 µg/kg); (C) chromatogram of control sultanas after one year of storage (0.19 µg/kg); (D) chromatogram of dipping pretreated currants after one year of storage (0.34 µg/kg); (E) chromatogram of control currants after one year of storage (0.21 µg/kg).

differences in OTA concentration between the pretreated and control groups during different storage periods. A difference with $p \leq 0.05$ was considered significant.

Results and Discussion

OTA occurrence in pretreatment and control groups OTA was not detected in the fresh grapes. Similarly, both pretreated and control dried fruits did not contain OTA before storage. Spore behaviors adhere to berries, and the germination and growth of the berries depend on the air temperature and relative humidity (23). The Turpan region is located in a low-lying basin, which has long hours of sunshine, a high daytime temperature, and cool nights during the grape development period. In addition, the Chunche chamber is usually built at a high slope and far away from the vineyard. Hocking's research showed that the incidence of black *Aspergillus* was high in vineyard soil and viable spores could be detected in the surface soil (24). The ripened grapes were hung on the wooden shelves in the middle of Chunche, with ventilated, cool, and relatively dry conditions. All these conditions in Turpan could prevent grapes from fungal infection and germination.

OTA occurrence during storage The present OTA accumulation study in sultanas and currants was investigated under real environment

conditions, which was under a more gradual change in temperature and humidity compared to other studies (16,25).

Both the control and pretreated groups in these two varieties started to be stored from October for up to one year. The OTA content of sultanas and currants in the two groups were detected every month, and the results are shown in Fig. 2.

It was observed that OTA did not exist in either of the groups at the beginning of storage for both varieties. This was because during this storage period, the temperature fell below zero (Fig. 3). It has been confirmed that the extreme growth temperature for most of *Aspergillus* is 7°C, while *Penicillium* can grow at a lower temperature of 5°C (26). Therefore, the extremely low-temperature conditions in Turpan's winter inhibited germination of the spores.

OTA was initially detected in the dipping pretreated sultana group in the fifth storage month (March 2013; spring period). This period had a higher temperature compared to the winter period (Fig. 2). The relatively high temperature might have induced the germination of spores. After the germination, fungi started to grow and produced OTA (27,28). Surprisingly, the control group was not contaminated until six months later than the dipping pretreated sultanas, and the content of OTA in the control sultanas was significantly lower ($p \leq 0.05$) under the same storage conditions. This suggested that the intact wax layer on the surface of the sultanas might have a protective function and delay the production of OTA by the germinated fungi. A similar finding was made for the currants. Only until the sixth month,

Fig. 2. The concentration of ochratoxin A in dipping treatment sultanas (◆), untreated sultanas (▲), dipping treatment currants (■), and untreated currants (●) during one year of storage. The capital letters A and B represent the difference between the dipping treated currants and sultanas after the same storage time ($p \leq 0.05$). The lowercase a and b represent the difference between the untreated currants and sultanas in the same storage time ($p \leq 0.05$). The concentration of samples with no detectable ochratoxin A was considered as $\text{LOD}^a/2$ ($0.035 \mu\text{g/kg}$) for the plot. ^aLOD=limit of detection ($0.07 \mu\text{g/kg}$).

Fig. 3. The temperature Shanshan region during one year of storage.

OTA was found in the treated currants group, while the control group was contaminated three months later.

In general, potassium carbonate-ethyl oleate has been reported to be capable of removing the wax layer of raisins and thus results in the formation of a micropore on the cuticle. This increased the drying rate during raisin production (19). However, the wax layer has been reported to provide a barrier to prevent against invasion of pathogenic organisms (29,30). Removal of the wax layer could destroy the integrity of the grape surfaces, causing fungi sporulation and/or infection. Guo *et al.* (30) found that removing wax from the surface of kernels greatly increased the accumulation of aflatoxin.

Therefore, fungi more easily formed colonies on the surface and further produced mycotoxin on the uncovered raisins. This could explain why OTA was detected in both of the dipping pretreated dried vine fruits much earlier than in the control. Khalesi *et al.* (31) carried out the modeling experiments of liquorice root storage. The temperature (T) and relative humidity (RH) was controlled to mimic autumn ($T=15^\circ\text{C}$, $\text{RH}=49\%$), winter ($T=9^\circ\text{C}$, $\text{RH}=51\%$), spring ($T=22^\circ\text{C}$, $\text{RH}=35\%$), and summer ($T=29^\circ\text{C}$, $\text{RH}=27\%$). They found that after 60 days of storage, the OTA contamination increased in the order: winter, autumn, spring, and summer. The OTA concentration under the summer-simulated condition was significantly higher than

Table 1. Hundred-grain weight, water content, Brix degrees, pH, and titratable acidity of ripe Thompson seedless and Flame seedless grapes

Grape variety	Hundred-grain weight (g)	Water content (%)	Brix degree	pH	Titratable acidity (g/L tartaric acid)
Thompson seedless	281.2	75.3	21.8	4.0	11.5
Flame seedless	170.5	75.5	22.3	3.8	9.6

in the other seasons. The results indicated that temperature played a more significant role on OTA formation in liquorice root than RH (31). We inferred that, although the humidity did not directly affect the accumulation of OTA, the surface of the dried vine fruit might absorb moisture in a high humidity condition. After rehydration, fungi more easily infected the dried vine fruit when the temperature increased.

For the treated samples, OTA was found in the sultanas and currants during the spring period and slowly accumulated in summer, while the OTA content of the currants rose more sharply than the sultanas during autumn. After one year of storage, the OTA level of the currants was significant higher compared to those of the sultanas ($p \leq 0.05$). For control samples, the currant was initially contaminated by OTA in summer and the content increased slightly during the storage period, while OTA was detected in sultanas during autumn and increased rapidly. After the storage period, the currants showed a higher OTA risk than the sultanas, but the difference was not significant ($p \leq 0.05$). The currant and sultana samples with the same treatment showed different OTA contents. The red variety of currants had earlier and more serious OTA contamination. This indicated that the components in the red dried vine fruits may favor fungal growth, which would lead to a faster and higher accumulation of OTA.

In the eighth month, the OTA concentration in the dipping pretreated groups decreased slightly, which was also observed in other studies (25,31,32). Among *Aspergillus* section *Nigri*, different strains of *A. carbonarius* or *A. niger* showed different degrees of toxin degradation (25,33).

After the one-year storage period, the OTA concentration was 0.22 ± 0.02 and 0.19 ± 0.01 $\mu\text{g/kg}$ in the dipping pretreated group and the control for the sultanas, respectively, and 0.34 ± 0.02 and 0.21 ± 0.01 $\mu\text{g/kg}$ for the currants, respectively. As we can see, the OTA content increased with increasing storage time, and the samples treated with dipping solution showed an apparent OTA increase. The OTA content in sultanas and currants (0.34 and 0.21 $\mu\text{g/kg}$, respectively) might not cause significant health issues, mainly due to the specific climate of Sinkiang, a much drier and cooler region in China. However, the dried fruits that underwent the dipping treatment exhibited higher potential hazards than the untreated ones when the commercial product was transported and stored in warm and humid areas. In our previous study, we found that higher OTA content in dried vine fruit was observed in the south regions with high temperature and wet climate properties. The OTA concentration detected from one sultana sample was 12.83 $\mu\text{g/kg}$, much higher than the European limit (11). Therefore, the storage conditions should be strictly controlled,

especially when storing dipping pretreated raisins. Specifically, a low-temperature, relatively dry, and well-ventilated environment should be used. The adoption of modified atmosphere packaging may be a good way to achieve this (34). In order to more fully elucidate the mechanism behind the accumulation of OTA in dried vine fruits during storage, future studies should be focused on the analyses of microbiological species, and the specific effect of ochratoxigenic fungi on OTA production should be further examined using biomass analyses.

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