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Measurement of the Patulin toxicant using high performance liquid chromatography (HPLC) in apple juices supplied in Khorramabad City, Iran

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ABSTRACT

Making use of low quality moldy and worm-eaten fruits for juice production causes various irritations in human body due to its hazardous compounds. Today, Patulin toxicant is one of the most important compounds to be investigated in juices, particularly in apple juices. This research aims to measure the amount of Patulin toxicant and identify the molding factors in apple juices supplied in Khorramabad shops. After preparing a list of shops supplying and selling h\juices in Khorramabad, 64 apple juices packs were collected at random. The Patulin measurement was accomplished using high performance liquid chromatography (HPLC) and the molding factors identification also was performed using macroscopic, microscopic and other necessary tests after the sample were cultured in standard method. Out of 64 sample investigated from presence of lack of mold perspective, 61 (95.3%) lacked mold and 1 (1.6%) had Aspergillus terreus mold and 2 (3.1%) had Penicillium mold*. The Patulin level measured in 31 samples (48%) was negative and in 33 ones (52%) was positive in range 5.102-26.484 µg.l^{-1}. The data obtained from samples was evaluated well in comparison to external standards and the correlation coefficient of 0.99 was indicated. The results obtained from this research indicated that the mean Patulin measured in apple juices studied was less than the EU and Iranian standards.*

Keywords: apple juice, mycotoxins, Patulin, HPLC, *Aspergillus*, *Penicillium*

INTRODUCTION

Patulin (PAT) is a secondary metabolic toxicant produced from different *Penicillium*, *Aspergillus* and *Byssochlamys* molds. This mycotoxin can be found in several juices such as apple, pear, grapes, apricots, strawberry, blueberries and peach juices and it often occurs in commercial juices such as apple. This variety of the juices causes a very significant impact on the society's health an economy state. The PAT consumption symptoms are fidgetiness, paroxysm, intestinal edema, ulcer, inflammation and vomiting [1].

Low level of Patulin in apple products is mostly focused due to their chronic and acute effects. Acute toxic effects of PTA in human includes nausea, vomiting, other digestive system damages and sever exposure can lead to cancerous tumors, genetic mutations and embryonic disorders [2]. Considering the fact that an acidic environment containing glucose with humidity of 70-90% is desirable for molds, hence juice is a favorable growth medium for these molds. Various kinds of the molds and yeast which are growing in the juices can secret mycotoxin which is poisonous and

lethal for human and animals [3, 4]. The EU; on one hand, has announced the maximum level of PAT for apple juice up to 50 µg per liter and for solid products 25 µgper liter and for children and infants' food products 10 µg per liter (Food and Agriculture Organization of United Nation (FAO) and WHO, 2002). The Patulin genetic toxicity studied on bacteria, fungus and mammalian cells have been conducted and their results implied mutation, chromosome abnormality, sister chromatid exchange and chromosome failure. Patulin bond covalently to thiol microtubules and causes their polymerization containment which depends on the concentration [5]. Patulin causes chromosome abnormalities such as DNA failure. Patulin's clastogenic effect in presence of 30 µg per liter of ascorbic acid is not observed [6]. Patulin causes that sister chromatid exchange increases in Chines hamster ovule (CHO-k1) and human lymphocytes and in doses more than 15 µM causes failure in DNA strands in human embryonic kidney cells (HEK 293) [7].

The main goal of present research is to measure the level of Patulin toxicant in apple juices sold in Khorramabad. Patulin measurement is performed using HPLC and identification of molding is accomplished after the sample cultures based on standard method using macroscopic and microscopic characteristics and other necessary tests.

MATERIALS AND METHODS

Undoubtedly, chromatography is the most important and most applicable separation method. Chromatography was discovered by Tissot, Russian herbalist, in early 20th century (1903). Also, there are various methods in order to determine the Patulin mycotoxin level including high performance chromatography, gas chromatography (GC) and thin layer chromatography (TLC) used [8]. Contrary to other mycotoxins, ELISA kits are not available commercially for Patulin [9]. In order to measure the Patulin in apple juices based on standard 7438, namely juice-Patulin determination in apple juices and its products using high performance liquid chromatography and tools, equipment and necessary materials mentioned, one would proceed on the following stages basis:

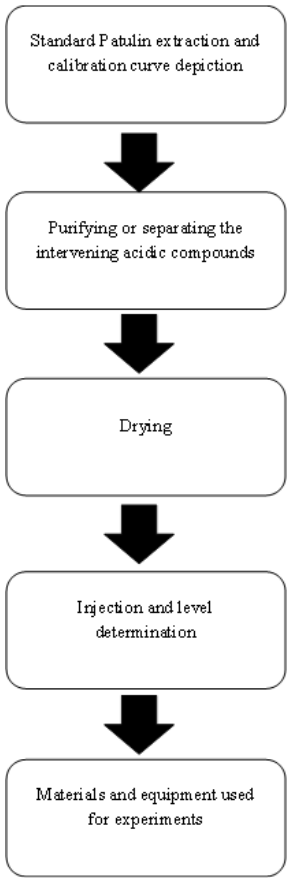

Figure1. Process stages

The above mentioned stages are explained as follow:
1. Twenty five ml of HPLC grade water was taken using pipette and transferred to clean and dry decantation funnel. Then using a 15 µl syringe the Patulin standard solution (50 µg per ml) was added to it. Then, using the clean and dry 25 ml pipette 25 ml of ethyl acetate was taken and transferred to the funnel and funnel was shaken by hand for 2 minutes. It was allowed for phases to be separated from each other. The down phase was disposed and up one was maintained in the decantation funnel.
2. Two ml of 1.5% sodium carbonate solution was added to the up phase and was shaken by hand for 15 s, well and then it was allowed for the phases to become separated. After being bi-phase, down phase was transferred to an Erlenmeyer flask and then disposed. The organic phase (ethyl acetate) was stored in a clean and dry 80 ml beaker and 2 or 3 drops of concentrated acetic acid was added.
3. The ethyl acetate and concentrated acetic acid containing beaker was stored in a bathroom with temperature of 40 ℃in order so that the remaining 3 ml volume evaporate. After drying, 2 ml of HPLC grade was added to the beaker and the beaker's wall was cleaned well.
4. Twenty µl of the solution was injected to the HPLC using a HPLC-specific syringe. The peak level obtained for standard Patulin was 50 $\mu g.l^{-1}$.
5. The experiment was iterated by concentrations of 20, 30 and 40 µg per liter of the Patulin standard solution and each time the level beneath the peak obtained from chromatogram was noted. The calibration curve was depicted based on the areas and the standard toxicant's concentration on the transverse and longitudinal axes of which the changes of the standard toxicant's concentration in term of µg/l and changes of the area beneath the curve or the spectrum's height were indicated, respectively (such that the area 0 to 40 µg/l of Patulin was quantified using the calibration curve).

In order to measure the Patulin in apple juice samples, the mentioned method was iterated and there were apple juice samples (25 ml of apple juice with brix of 11.2) used and the Patulin amount was calculated from the calibration curve. The spectrum obtained was compared with the standard one from retention time perspective and the contamination level was calculated from calibration curve. Patulin level determination was performed by measurement of the area beneath curve in retention time of Patulin and its comparison with calibration curve. To do so, after the sample size was determined by a statistics expert (n=64), first the apple juices produced in domestic factories from different brands and production date in both summer and winter were samples at random from the supermarkets in Khorramabad and then were used in order to analyze the Patulin and identify the molding factors. All samples were stored in refrigerator in temperature of 4 ℃ during the tests. This study was conducted in cooperating laboratory of Industry and Culture Corporation of Takdaneh juice in Marand, east Azarbayejan in order to measure the Patulin toxicant level in apple juice samples.

Materials used
Necessary materials for chemical tests in present research included:
- Distilled water suitable for liquid chromatography (HPLC Grade) based on the grade 1 Iranian national standard, No. 1728
- Ethyl acetate suitable for liquid chromatography, German MERK company
- Water free sodium carbonate, German MERK company
- Glacial acetic acid, German MERK company
- Acetonitrile suitable for HPLC grade, German MERK company
- Standard Patulin, German MERK company

Equipment used fin chemical test
In addition to typical laboratory equipment, other tools and equipment are as follow:
- Glass tools including graded cylinder of 50 and 100 ml volume, over cup balloon of 25, 50 and 100 ml volume, separating funnel of 100 ml volume, glass beaker of 80, 100 ml volume, head-screwed or over cupErlenmeyer of 100 ml volume, head-screwed test tube of 5, 10 ml volume and sampler of grade A were provided.
- Syringe head filter with aperture of 0.45 µm diameter and filter paper with glass fibers and aperture diameter of 1.6 µm (or smaller)
- Seven Liter boiling water bath, model WNB7, made in German MEMMERET, regulated in 40 ℃.
- Ultrasonic, model MISONIX, made in USA
- Scale with accuracy and sensitivity of 0.0001 g, model 224 QUINTINX, made in German Sartorius
- High performance liquid chromatography device

RESULTS

Figures 1- 3 demonstrate the Patulin standards solution chromatogram with concentrations of 40, 30 and 20 µg/l, respectively. Considering the goal of the research in relation to investigation of the Patulin presence and identification of the molding factor in apple juices sold in Khorramabad, first the Patulin standard solution was prepared by different concentrations (20, 30 and 40 µg/l) and then using HPLC, in order to identify and diagnose the compounds in chromatogram the analyses performed, qualitatively. Since the retention time for each material is constant in a laboratory particular situation and system, one can make use of it to determine the type of Patulin. To do so, the chromatogram of the analyze (Patulin in apple juices) would be compared to the chromatograms obtained from the analyte's standard solution (Patulin). In case the time retention is similar, one can certainly determine the type of material (Patulin)

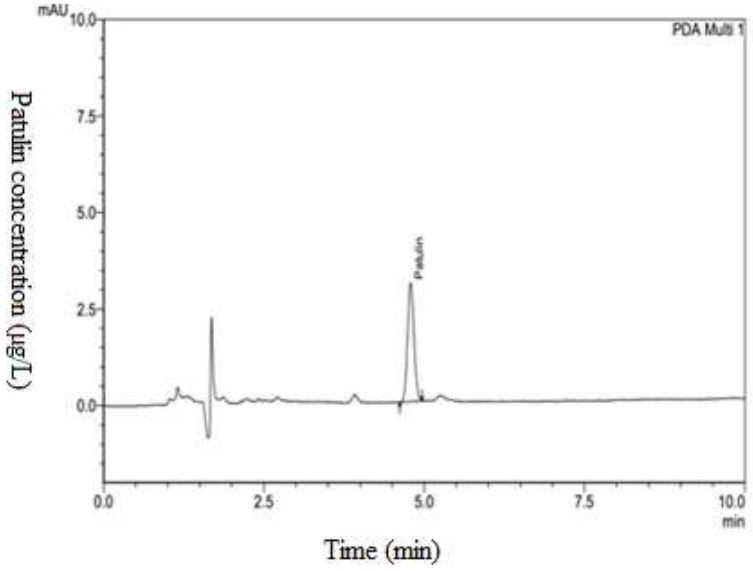

Figure 1. Patulin standard solution chromatogram with concentration of 20 µg/l

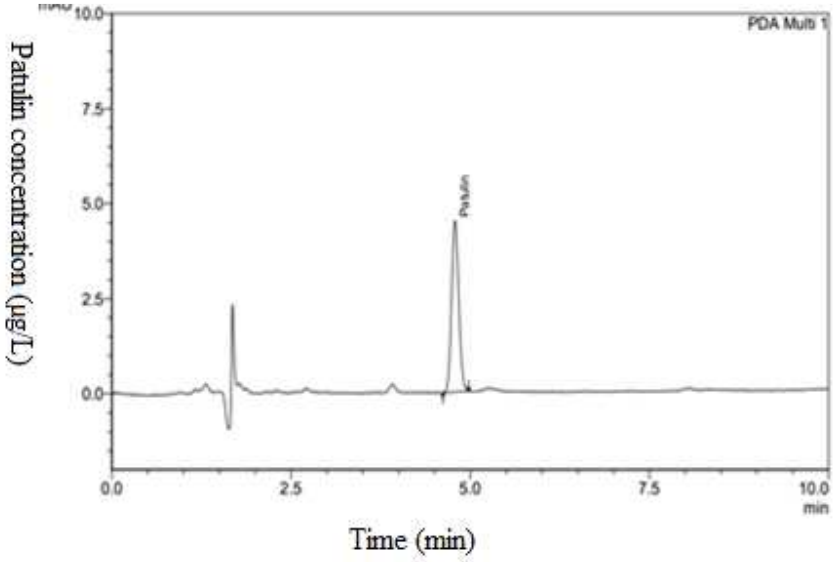

Figure 2. Patulin standard solution chromatogram with concentration of 30 µg/l

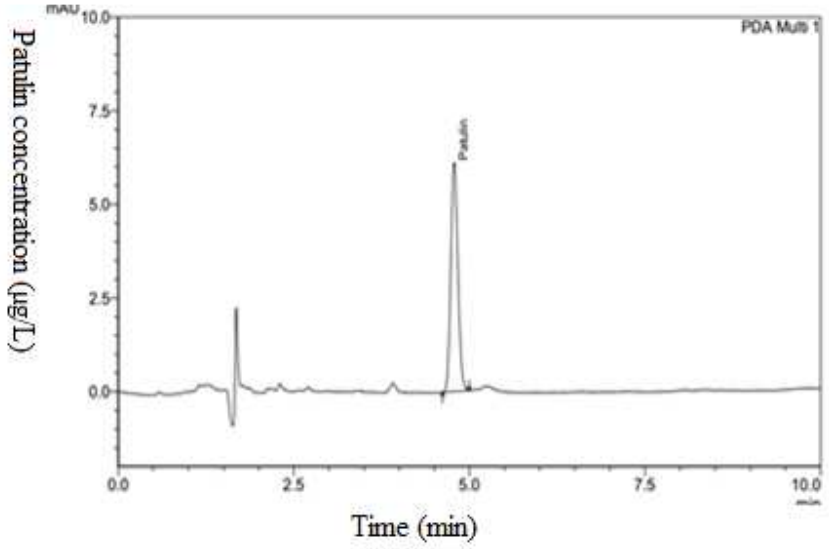

Figure 3. Patulin standard solution chromatogram with concentration of 40 µg/l

Table1. Concentration, area and retention time of the Patulin standard solution used

Patulin analyte	Concentration µg/l	Retention time	Mean area	Area
1	20	4/781	21435/7	21436
2	30	4/781	31520/7	31521
3	40	4/781	43006/7	43007

As it is seen from Table 1, retention time in chromatograms of the Patulin standard solutions is 4.781 min with known concentrations which can be used in order to identify the passive Patulin peak in apple juice samples.

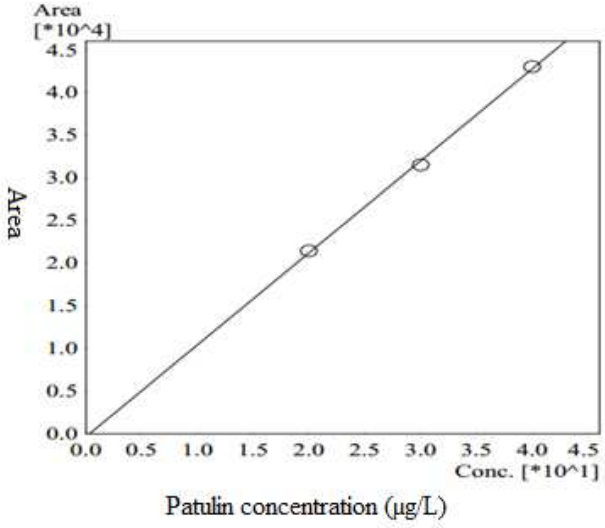

Figure 4. Patulin calibration in concentration area of 20-40 µg/l

*1078.55*x-368.849=f(x)*

Rr_1=0.9992976; Rr_2=0.9985958

Mean RF:1065.88; RFSD: 13.2644; RFRSD: 1.24445

Relation 1 expresses the line equation obtained from calibration curve where *f(x)* is the area beneath peak and *x* is the Patulin concentration (Figure 4). Now, in order to quantify the analyze (Patulin) level the qualitative analyses are performed and using external standard, by giving different Patulin standard solution concentration made to the HPLC, using area beneath peak or height obtained from the chromatograms of these standards, calibration curve

(height or area beneath peak curve, in term of concentration) was depicted and using the line equation obtained, the height and/or area beneath peak of passive sample, the accurate amount of the analyze (Patulin) was calculated.

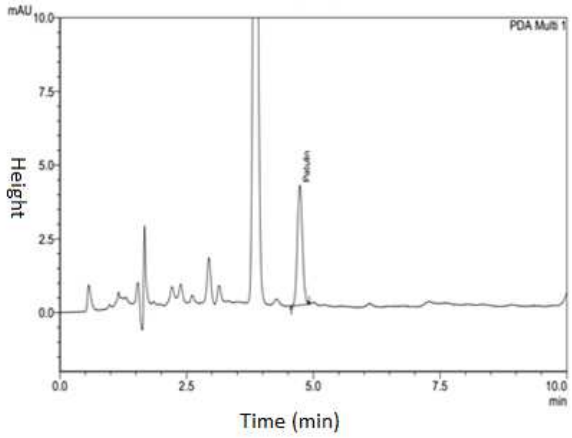

Figure 5. Chromatogram of 20apple juice samples, investigated with Patulin concentration of 26.484 µg/l

The chromatogram related to the apple juice samples studied using HPLC device indicates the column C18, particle size of the 3µm and column dimensions of 100mm×4.6 mm, mobile phase flow rate of 1 ml/min, detector PDA with wavelength of 276 nm, injection level of 20 µl. The Patulin concentration in apple juice after substitution of the area beneath the peak in the line equation obtained from calibration curve would be calculated from *f(x)=1078.55*x-3680.849* (relation 1) equal to 26.484 µg/l. which is lower than the allowable level by EU and Iranian standard (50 µg/l) (Figure 5). About samples 17, 18 and 19 also the chromatogram and retention time, area beneath the peak and Patulin concentration would be determined. In order to analyze data the SPSS software, version 20, bar diagram with error of 95% reliability distance for mean and independent t-test or its nonparametric equivalent, i.e. man Whitney were used.

Table2. Patulin concentration in apple juices studied

Sample	Number	Patulin concentration (0 µg/l)	Patulin concentration (1-5 µg/l)	Patulin concentration (5-10 µg/l)	Patulin concentration (10-20 µg/l)	Patulin concentration (20-30 µg/l)	Patulin concentration (30-50 µg/l)	Patulin concentration (>50 µg/l)
Apple juice	64 (100%)	31 (44/48%)	24 (37/5%)	4 (6/25%)	3 (4/69%)	2 (3/12%)	0 (0%)	0 (0%)

Considering the Patulin concentration levels in apple juices contaminated, in range 1.425-26.484 µg/l it is varying and significantly lower than the allowable 50 µg/l limit. As it is seen from table 2, the measured Patulin level in 64 samples of apple juice studied in concentration area less than 5 µg/l, the most contamination by Patulin was 24 samples (37.5%), in concentration 5-10 µg/l 4 samples (6.25%), in 10-20 µg/l, 3 samples (4.69%), in 20-30 µg/l 2 samples (3.12%) were indicated which are less than allowable level.

Table3. Patulin concentration in apple juice samples contaminated by Patulin (33 samples)

Apple juice sample	Concentration µg/l	Apple juice sample	Concentration µg/l	Apple juice sample	Concentration µg/l
1	4/76	12	8/007	23	1/776
2	4/785	13	3/495	24	2/838
3	2/297	14	1/425	25	3/495
4	4/8	15	2/67	26	8/007
5	3/526	16	1/921	27	3/131
6	3/131	17	17/755	28	1/935
7	1/776	18	13/804	29	7/22
8	1/425	19	23/051	30	2/838
9	7/22	20	12/029	31	2/67
10	1/953	21	26/484	32	1/97
11	1/97	22	1/921	33	3/526

The results of the Patulin measurement are listed in table 3. As it is seen from table, Patulin concentration in 64 samples of apple juice studied, it was varying in 33 samples contaminated by Patulin with concentration of 1.425-26.484 µg/l. the lowest level of the Patulin related to code 8 and 14 with 1.425 µg/l and the highest level related to code 21 with 26.484 µg/l which were less than the allowable level in comparison to EU and Iranian standards (50 µg/l). In other word, it is significantly less than the allowable level (50 µg/l).

Table4. Comparison of frequency and presence or lack percentages of Patulin in apple juice samples

Apple juice samples		Frequency	Percentage
Negative	Lacking Patulin	31	48/44
Positive	Contaminated by Patulin	33	51/56
	sum	64	100

As it is seen from table 4, out of 64 samples of apple juice investigated from presence or lack of Patulin perspective, 31 samples (48.44) lacked Patulin and 33 (51.56) were contaminated.

Table5. Comparison of Max, Min, mean Patulin concentration in apple juice samples studied

Descriptive statistics	Sample size	Min	Max	mean	SD
Concentration (µg/l)	64	0/0000	26/4840	2/962953	5/2857033
Area	64	0	28195	2948/12	5392/640
height	64	0	4066	437/59	789/831

Results of the table 5 indicate the max, min, mean, Patulin mean concentration in samples studied which are significantly less than the Iranian and EU standards (50 µg/l) and have significant difference ($P<0.05$).

DISCUSSION

In term of toxicity hazard evaluation of different Patulin such as chronic (gastrointestinal inflammation, nausea and vomiting, edema, fidgetiness, paroxysm etc.), acute (mutation, teratogenicity, immunity system weakness etc.), and cell toxicity (protein synthesis and DNA inhibition, sodium and potassium pomp inhibition etc.) are considered. Food and drug administration's (FDA) stated that the daily maximum tolerable doses for Patulin would be 0.43 µg/l for each kg of body weight. FDA and WHO determine the allowable level of Patulin as 50µg/kg for apple juice. In majority of the countries such as Iran, the allowable level of this toxicant in apple juice is µg/kg. Delavar et al. [10] in their research made use of the HPLC in line with determining the Patulin level in apple juices. This is consistent with present study and indicates that the results of both studies are in a line. Fathi Achachlui et al. [11]also in their research determined that the reported level of Patulin in apple juices in northwest Iran is more than the allowable level which is in contrast to the observation in west of Iran (Khorramabad). Rahimi [12] in his research investigated the effects of Patulin and noted that this toxicant is observed in juices such as pear, apple juices etc. This issue indicates the suitable selection of investigated sample in present research. On the other hand, Bracket et al. [13] observed in their investigations that Patulin contamination of apple juice can be more than the allowable level even to 800µg/l. Prieta et al [14] in their research investigated the Patulin in apple juice and stated that level of this toxicant in apple juice is prevailing in Spain extensively. This is why we conducted the present research. In work by Sylas et al. [15] it was detected that the highest level of the Patulin has been observed in crumbling juices like apple one which is consistent with our results. Spadaro et al [16] detected the Patulin level in apple juice in Italy less than the allowable limit. Welke et al. [17] in their research considered the lower level of the Patulin in apple juices than global standard (50 µg/l) which is also in good agreement with our results. Also, Ionescue et al. [18] in their research investigated the different concentrations of the Patulin in apple juices which gained results consistent with ours. In other research, Milicevic et al. [19] stated that Patulin is one of the Mycotoxins present in juices and fruits which are life-threatening for human being. Al-Hezmi [20] also in his research reported that out of 17 types of the juices, 1 has more than allowable level the Patulin which is in agreement with our results. In other research by Catana et al. [21] in Romania the Patulin level in apple juice was investigated using HPLC method and the results implied that the level of the Patulin was lower than the allowable one in apple juice. The results stated in this research are in line with our results. Forozan and Medadlu [22] investigated the Patulin level in their research in apple juice. Their results also are in agreement with present research and indicated that there are few samples with higher Patulin than allowable dose.

CONCLUSION

The results of the sample analyses in present research indicated that the level of Patulin in apple juices in Khorramabad shops is less than the FDA and WHO limits and its level in some cases also was negligible. This result probably can be due to making lower use of natural juices in these drinks. Among effective factors on mycotoxins presence in foods one can point to the conditions under the environmental and biological control related to food storage. Other external factors such as climate and internal factors such as mold characteristics, pressure, diversity and instability of the properties produce toxicants which are difficult to control. The best procedure to fight the molds secreting Patulin is to regard the health principles from the farm to the production process in factory. What is clear is that one can reduce the Patulin level during the apple juice processing by separating the molded fruits, washing them, purification of the juice using vacuum filter, juice fermentation, UV beam. In order to have a healthy and mold toxicant free product, the issue can be investigated in 2 aspects; one is identification of the Patulin production and methods to prevent from it and other is to identify the purification of the contaminated products. One of the most important Patulin producing factors can be temperature, humidity level, oxygen concentration, substrate type, pH of the food, microbial interactions, presence or lack of inhibitors such as organic acids and mechanical damages. It is obvious that controlling the mentioned factors accurately can prevent to large extent from Patulin production. The most important mechanical factor is impact which can destroy the food cell tissues. Fruits which damaged during transference would become brown and then decayed when being stored in refrigerator or in retails due to the destruction and tear down of their cells. The damaged parts let the molds and bacteria to penetrate the fruit and lead to its decay. It seems that the following methods can be helpful in this regard:

- Making use of new packaging by juice containers using novel technologies such as Nano-containers
- Complete supervision on all production chain levels and storage of the juices and environmental conditions by supervision institutions
- Making use of new methods in order to eliminate the Patulin mycotoxins in juices such as making use of high hydrostatic pressure for deactivation of micro-organisms
- Applying sample methods for refining the mycotoxins contaminated juices
- Correct, accurate washing and making use of high-pressure water

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