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Determination of Patulin in Apple Juice Produced in Iran, HPLC with Diode Array Detection

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Abstract: Patulin is a toxic compound produced by a number of fungi of genera (*Penicillium*, *Aspergillus* and *Byssochylamys*). It has been formed as a result of 'blue mold rot' on fruits, such as apple, grape, pineapple, banana, orange, pear and apricot during growth, harvest and storage which subsequently transferred to the apple juice and derived products, such as concentrates and so on. Researches demonstrated broad spectrum of toxicological properties as mutagenic, immunotoxic, neurotoxic and causing adverse effects on the health. The maximum permitted concentration of patulin in food stuffs has been set at 50µg/l by the world Health organization and Iran's standard institute (standard number 5925). This survey was conducted to determine patulin content (mycotoxin), which holds significant adverse health risk to consumers, in three leading apple juice concentrate manufactures in Iran. In this study 32 apple juice concentrates, belonged to three staple factories, were purchased from different shops located in Karaj. Mycotoxin wax extracted by using ethyl acetate and sodium carbonate and the level of the patulin measured by HPLC. Mean concentration of patulin were 69.7±79.49 µg/l and contamination ranged from 16.8 µg/l to 431 µg/l and approximately 40.6% of all apple juice samples contained patulin level higher than 50 µg/l. The mean contamination of patulin in 1, 2, 3 factory products were detected respectively: 53.7±36.7, 100±116.49, 57.39±61.9 microgram per liter and 40.6.1, 50, 22.2 percent of products manufactured in these factories demonstrated fairly high incidence of patulin. As a result, this survey indicated high contamination in Iran's apple juices which holds significant adverse health risk. In order to reduce the incidence and the level of patulin contamination, more inspections are required and individuals who are involved in, must be trained and be aware of the cryptic risk of patulin (mycotoxin).

Key words: Toxic compound · Patulin · HPLC

INTRODUCTION

Patulin (mycotoxin) has been found to be a natural contaminant of processed apple products and its presence was suggested to be an indicative of the quality of the fruit used in production [1]. It is particularly produced by various rotten funguses as a secondary metabolite and due to its cyclic and varied molecular structure is carcinogenic [2]. Patulin also comes from a kind of mycotoxin group and nowadays the level of patulin in fruit juices is a major concern [3]. It is not only plays an important part in the quality of fruit juices, but also is an index in distinguishing how much damaged and rotten fruits have been applied in production process [3,4]. Several factors have influences on the level of patulin, produced in apple juice, such as:

- Using apples found on the ground (falling down the trees).
- Paying no attention to harvest properly, being usually performed by gardeners, thus most apples are contaminated by the fungi generating palutin.
- Being infected through transportation system.
- Disregarding sorting out (isolating rotten apples) processes in factories give rise to the any mention of patulin [5-7].

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According to food safety management system known as hazardous analysis critical control points (HACCP), being a profitable method and preventing safety risks in food stuffs, we may reach desirable point in producing healthy hygienic apple juice which contains lowest level of patulin [8, 9]. Exporting concentrate is growing significantly in recent years and our dear country is one of the major apple juice concentrate exporter in the world. As a consequence products maintaining optimal quality at lowest prices are demanded. The evaluation on the contamination with patulin in apple and apple juices become an important factor to guarantee the quality [9-11]. Whereas this article showss that apple juice concentrates produced in Iran contained high rate of patulin with concentration of over 40.6% which indicates the need for raising awareness about patulin contamination in individuals who are involved in apple juice industry [12, 13, 14].

MATERIALS AND METHODS

Sample Preparation: 32 samples of apple juice concentrate were collected from different department stores located in Karaj. Samples, labeled in various season and date (exclude november and december - no samples, were manufactured in these months), had been produced by three leading concentrate producers in Iran. Samples stored below -4° in refrigerator, maintain them under the same conditions, before analysis. At the time of conducting experiments, they were placed outside to reach room temperature [12-14]. All samples were extracted as described below and analyzed by HPLC [15].

Extraction and Cleanup: In order to get samples ready to be analyzed, all apple juice packages were cut and their contents poured through a separator funnel, covered by 0860 Wattman paper filter on the top, into 250 ml Erlenmeyer. Each ml of apple concentrate (having 70° Brix) was diluted to 50 ml distilled water, done by refractometer, so we had apple juice with Brix 12 then 10 ml clear apple juice (now having Brix 12) was sequentially extracted with 10 ml portion of Ethyl Acetate, as a drying agent, in a clean separate funnel and vigorously shaken for 1 minute using vortex mixer, putting separator funnel aside for a while [16, 17]. Two phases were separated and the upper layer was transformed into culture tube. Pooled Ethyl Acetate fractions were washed in the same separator funnel with 1 ml of aqueous Na2Co3 solution (Merck, Germany) and shook for 15 seconds, again two phases were separated. 1 ml diluted water added to the separator funnel, stirred for 15 seconds [18].

The organic separated phase (ethyl acetate) poured into dried clean 25 ml Erlenmeyer and was acidified by adding 2 drops of acetic acid (Merck, Germany). The organic solvent was removed by rotary evaporation in a water bath at 40±1° under a gentle stream of nitrogen. Some jelly form substances were formed in the bottom of Erlenmeyer which contains patulin. The residue was dissolved in 1 ml diluted water, rotated in a circulation shape then and injected into the column of HLPC (High performance liquid performance chromatography) preparation of standard curve [19, 20].

Validation of Analytical Method: Patulin standards concentration (50, 100, 200, 400,600 μg/l), were used to validate the accuracy of our expriments in testing apple juice samples (without patulin), which were "artificially" contaminated [21, 22].

It was prepared by dissolving 50 mg of pure crystalline patulin (sigmaaldrich-American) in appropriate ethyl acetate solution that we gain concentration of 20 mg/l. of patulin then 25 μ/l of this standard solution dissolved in 10 ml distilled water and entered through a separator funnel into Erlenmeyer flask. From now on, treated like an unknown sample and went through process which described formerly and injected to HPLC device [22, 23]. Then we had a diagram with a peak area. The same procedure repeted with using various standard solutions with 50, 100, 200, 300 μ/l patulin with concentration of20mg/l and each time obtained different peak area.

Measurement of Patulin by HPLC: Determination of patulin contamination was performed by high performance liquid chromatography. (perkin Elmer Zoo, America) HPLC analysis conditions:

- Mobile phase: acetonitrile, water,
- UV-VIS detector, λ=276nm,
- Injection Volume; 20 μ/l [23, 24]

Applying the optimum chromatographic conditions, analysis of patulin being in apple juice samples were carried out on a reversed phase c18 with UV detection at 276 nm. After reckoning peak area, acquired 20 minutes after injection, known as retention time, by total chrom software (special software attached to HPLC device). All the acquired data were transferred to excel software obtaining a linear function (equation y) which represents peak area (MAU) and X which indicates concentration (ppb).

Statistical Analysis: The process of evaluation was performed, using Excel software thus the mean concentration of patulin and standard deviation for three factories all together and separately (for each factory) calculated.

Results Obtaining Preparation Standard Curve: By using six different patulin standard solutions (in the rage of 0 to 600 ppb), calculation peak are, retention time (about 20 minutes after injection) and computation by particular HPLC software, the following value were observed which is presented in the following Table 1.

Data were transmitted to Excel software and linearity was observed with concentration up to 600 ppb. Standard curves (y = 189.35×-6054.9) were obtained and depicted.

In the survey conducted on 32 samples owned three outstanding concentrate makers in Iran, indicated that 40.6% of all samples were contaminated and contained over standard limit (50 μg/l). Also mean concentration of patulin measured to be 69.52±79.49 μg/l.

Diagram of general evaluation of patulin in 3 producing juice factories Samples, exceeding 50 μg/l are colored by red.

By monitoring results obtained from this study, we were able to determine confined area which embraced 99% of mean statistical figures. So that maximum rate (105.5 μg/l) as well as minimum rate (33.4 μg/l) lies in this diagram. The maximum rate and counter point (minimum rate) were computed.

The samples collected from the first factory, as shown in Table 2, were found to be positive (exceeding permitted limit) in 46.1% of all apple juice concentrates, ranging from 17 to 139 μg/l. The highest concentration (139 μg/l) was obtained from sample purchased in 23.10.2010 The total patulin level revealed by this method was 53.7±36.7 μg/l

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The result of patulin contamination in samples obtained from factory number two.

The samples obtained from the second factory, depicted in Table 4, turned out to bearing higher patulin concentrations than the acceptable limit in 50% of apple juice products.

Table 1: Peak area of patulin in standard samples

Peak area	Patulin concentration is standard sample (μgl)
0	0
3412.5	50
12880	100
32621.12	200
68071.24	400
108360.4	600

Table 2: Patulin level in samples obtained from factory number one

Sample number	Producation date	Patulin level	Exceeding 50μg/l
1	16.10.2010	81.29	+
2	02.02.2010	54.05	+
3	23.02.2010	139	-
4	07.03.2010	21.1	+
5	16.03.2010	71.01	+
6	14.04.2010	22.17	-
7	16.05.2010	19.36	-
8	27.05.2010	109.37	+
9	02.06.2010	17.41	-
10	17.06.2010	20.34	-
11	30.06.2010	62.07	+
12	15.07.2010	45.03	-
13	04.08.2010	36.12	-
		Mean=53.7±36.7	Total (+) 46.1%

Table 3: Patulin level in samples obtained from factory number one

Sample number	Production date	Patulin level	Exceeding 50μg/l
1	25.08.2009	137.8	+
2	23.01.2010	36.2	-
3	04.02.2010	105.99	+
4	28.02.2010	25	-
5	16.03.2010	69.01	+
6	30.03.2010	106.18	+
7	24.04.2010	431.58	+
8	15.06.2010	33.3	-
9	02.08.2010	30.5	-
10	18.08.2010	34.3	-
		Mean=100±116	Total (+) 50%

Table 4: Patulin level in samples obtained from factory number one

Sample number	Production date	Patulin level	Exceeding 50μg/l
1	05.10.2009	229.35	+
2	17.10.2009	56.3	+
3	22.02.2010	18.42	-
4	16.03.2010	16.81	-
5	25.04.2010	31.4	-
6	06.05.2010	39.8	-
7	10.05.2010	42.17	-
8	28.06.2010	37.61	-
9	01.10.2010	44.65	-
		Mean=57.39±61.9	Total (+) 22.2%

The mean patulin concentration was 100±116.49 μg/l. Maximum rate of contamination (431.58 μg/l) acquired by specimens bought in 24.04.2010 and lowest on 25 μg/l belonged to sample gained in 28.02.2010.

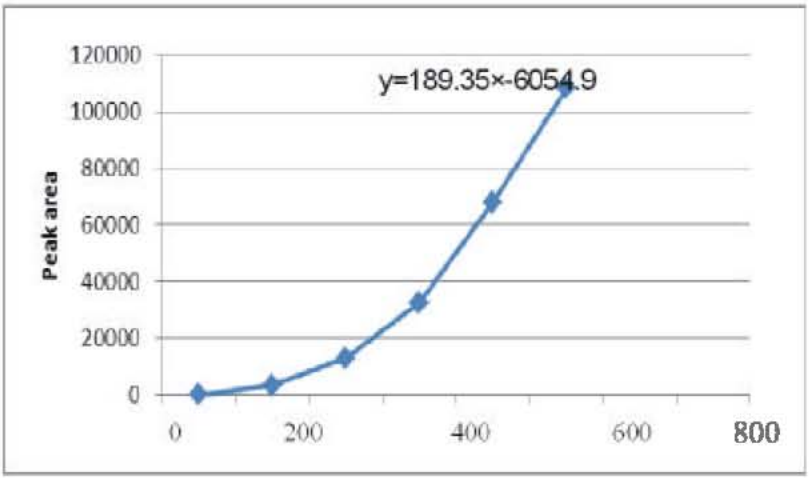

Fig. 1: Standard concentration of patulin general outcome of patulin concentration measurement

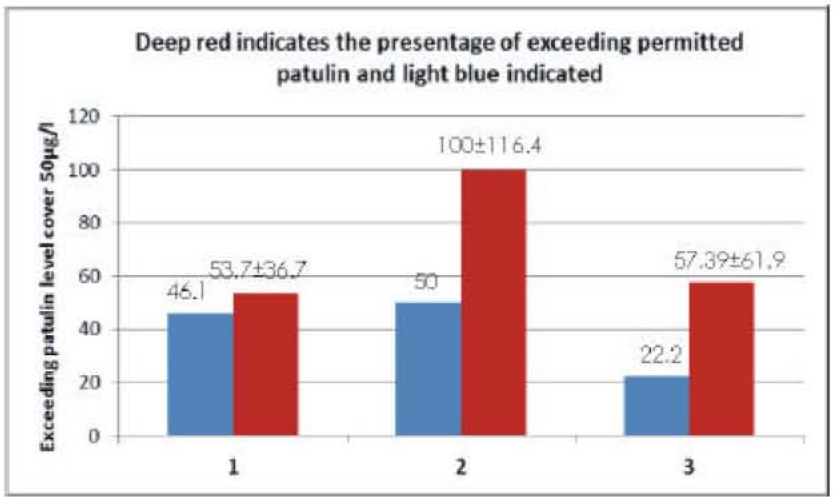

Fig. 2: Comparison of three factories the outcome of determination of patulin in samples collected from factory number one

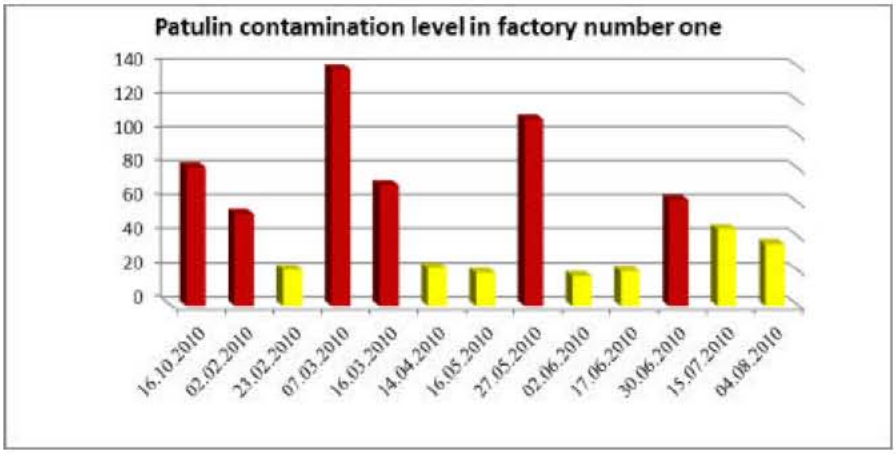

Fig. 3: Comparison of patulin level and product date

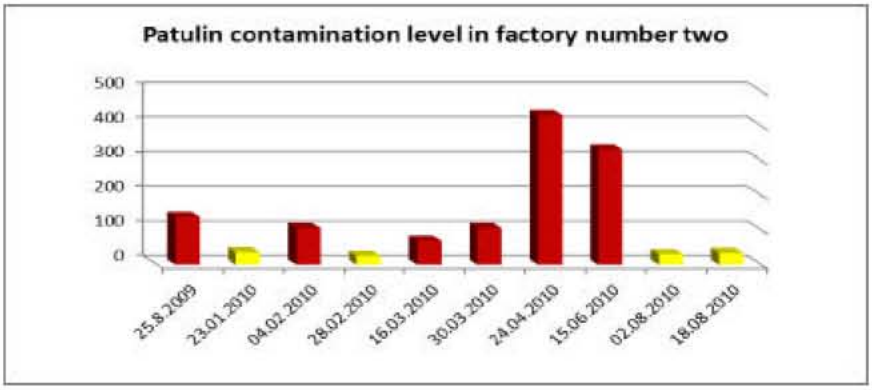

Fig. 4: Comparison of patulin level and product date the result of patulin level in samples gathered from factory number three

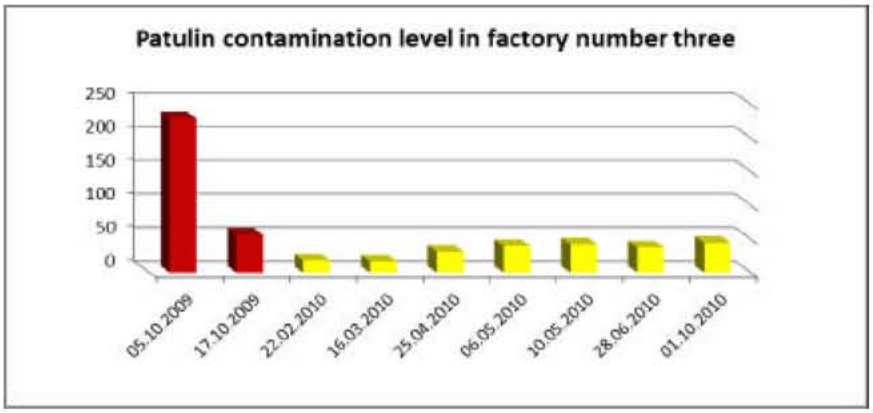

Fig. 5: Comparison of patulin level and product date

DISCUSSION

Patulin, is a member of compounds known as mycotoxins. It is a secondary metabolite produced by a wide range of fungi. Patulin has mainly been found in rotten, damaged parts of the fruits, particularly in apples and apple products and occasionally in other fruit such as pears and grape. Patulin possesses potential risk to health due to its genotoxicity, teratogenicity, immuno- toxicity effects and the amount of it must be lower than 50 µg/l, which is accepted globally, in fruit juices.

It is important to note that apple juice and apple juice concentrate is the most consumed juices, afterwards orange juice, in the world. Therefore it is considerably significant in economics. In addition, owning to its sweet taste it is much demanded by child hood. In this article we presented result of performed experiments done for the determination of patulin level in apple juice concentrate produced in various date by three leading manufactures, through high performance liquid chromatography. Contamination levels of Patulin were in this manner (Maximum rate, minimum rate):

- The first factory: 139 µg/l, 17.41 µg/l.
- The second factory: 431.58 µg/l, 25 µg/l.
- The third factory: 224-35 µg/l, 16.81 µg/l.

Similar manifestations in apple juices were reported by Achacheloe and his co-workers [3]. They explored the present of patulin obtained from six different factories lie in Northwest of Iran. Highest level of patulin belonged to apple juice concentrate, by the rate of 110.25 and 450 µg/l for red grape juice. According to the survey some of improved techniques for the production of apple products are required in order to reduce the incidence and level of patulin contamination in apple juices.

Patulin was detected in 3 samples that belong to only apple juice concentrate, owned the factories placed in northwest of Iran, exceeded permitted level and contamination rate were about 89.4, 95.34, 110.25 µg/l.

In our survey patulin also surpassed the normal limit in products manufactured three factories, number 1, 2, 3 and were 46.1, 50 and 22.2% respectively.

Inquiry done by Cheraghali and his group [5] on 42 apple juice and 23 apple juice concentrate in 1384, indicated that 33% of the apple juice and 56% of the apple juice concentrates possessed patulin higher than 50 μg/l.

In our survey, samples come from three factory exceeded patulin level (50 μg/l) were respectively 46, 50 and 22.2% as well as mean concentration of patulin were 53.7±36.7, 100±116.4 and 70±110.4 μg/l.

Mean concentration of patulin in apple juice samples surveyed, in our research, Cheraghali and cooperators, Achacheloi and his group [3], were slightly - Except the third factory which was about 5 times over safety limit - higher than maximum level recommended by world health organization. Whereas inquired previously done by Canadian food organization known as CFIA,s on imported on apple juice and apple juice concentrate demonstrated that only 15.9% of all samples obtained from nine varied countries were fairly over $10\frac{Mg}{L}$ and mean patulin concentration were $15.9\frac{Mg}{L}$, other identical investigation conducted on the apple juice concentrates presented that only 8.33 of them contained patulin relatively more than $10\frac{Mg}{L}$ and mean patulin concentration measured to be about $15.4\frac{Mg}{L}$. Compared to other nations, patulin concentration in apple juice and derived product that are manufactured in Iran are relatively high.

It is concluded that apple juice marketed in Iran contain rather high concentration of patulin which indicates the need for raising awareness about patulin contamination in individuals who are involved in apple juice industry. And this goal (reducing level of patulin) is gained by:

Putting more effort into promoting techniques for the production, qualified workers who are familiar with potential risk of patulin to health Health organization must have more control and regulations over producing process.

REFERENCES

1. Abolabas, C., 2005. Mollecular and cellular immunology. Tabib Publication.
2. Emamis, M., 1998. Medical Mycology, Tehran Univercity Publication.
3. Alameh, A. and M. Arzaghi Abyane, 2001. Mycotoxin Imamhossein Univercity Publication.
4. Fathiachacheloei, B., S. Azadmard, G. Hesari and M. Nemati, 1988. Patulim concentrate, thesis (master of degree), Tabriz Univercity).
5. Fathiachacheloei, B., S. Azadmard, G. Hesari and M. Nemati, 2009. Patulim concentratio in fruit juiced produced in weaternorth factories, scientific research Journal of Agriculfure Published by Tabriz Univercity, pp: 1-13.
6. Mohamad, K., 2009. Statisfical methods and hygenic markers, Banyad Publication.
7. Nazari, F., F. Ebrhimi and T. Faramarzi, 2009. My Cotoxins, Iran Uunivercity of Medical Scieces, Food and Drug Assistance, Article.
8. Agriculture and Consumer Protection, 2003. Manual on the application of the HACCP system in mycotoxin prevention and control, FAO Corporate Document Repositry, 73: 245.
9. Bado, E., 2007. Intralaboratory optimization and validation of a method for patulin determination ingrapes by. Thin-layer chromatography, Brazilian Journal of Microbiol., 38: 304-308.
10. Biing-hui, A. and L. Ting-Shun, 2003. Evaluation of genotoxic risk and DNA Damage in Mammalian Cells Exposed to Mycotoxin, Patulin in Apple Juice Product Food Safty Actin Plan Report 2008-2009.
11. Cheraghali, M., 2005. Incidence of Patulin Contamination in Apple Juice Produced in Iran, 16: 165-167.
12. Ingam, C.L., 2004. Survival of penicillium exansum and patulin production on stored apples after wash treatment, Journul of Food Chemistry and Toxicol., 69: 669-675.
13. Kawamoto, Y., 2008. Development and single laboratory validation of a method for patulin determiniationin fruit juices, World Mycotoxin Journal, 3: 59-65.
14. Kerri, H., 2009. Patulin surviellnce in apple cider and juice marketed in micigan, Journal of food protection wash treatment, Journal of Food Chemistry and Toxicol., 72: 1255-1261.
15. Lai, C., 2000. Detection of mycotoxin patulin in apple juice, Joornal of Food and Drug Analysis, 8: 85-96.
16. Lehman, P., 2007. Patulin influences the expression of Th1/Th2 cytokines by activated pripheral blood mononuclear cells and Tcells Through Depletion of Intracellar Glutathi.
17. Morales, H.H.B., 2008. Effects of apple and pear vaieties and PH on Patulin.

18. Manfred, M., 2005. Mutagenicity of the mycotoxin patulin in cultrred chinese hamster and its modulation by intracellular glutathione, Archives of Toxicol., 79: 11-121.
19. Oliveira, A. and J. Rueff, 2000. Induction of micronuclei and chromosomal aberrations by the mycotoxin patulin in mammalian cells, Mutagenesis, 15: 229-234.
20. Paul, O., 2010. Biosynthesis and Toxicological Effects of Patulin, 2: 613-631.
21. Perkinns, V., 2009. An interdiscipliary approach to reducing patulin in food through novel sensor development fungal identification and new processing methods, University of Main.
22. Rychlik, M., 2005. Quantification of the mycotoxin patulin in foods, Nutrition, 29: 9-15.
23. U.S. Food and Drug Administration, 2000. Patulin in apple juice, Apple Juice Concentration Andapple Juice Product, pp: 1-12.