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Incidence of Patulin in Apple Juices Produced in West Azerbayjan Province, Iran

Sh. Forouzan[1*], and A. Madadlou[2]

ABSTRACT

Food safety is a global concern due to an increasing awareness of consumers concerning exposure of foods to chemicals and contamination with such hazardous biochemicals as dioxins, mycotoxins, pesticides, polycyclic aromatic hydrocarbons, drugs and hormones. Patulin is one of the most injurious mycotoxins produced by a variety of molds, particularly *Aspergillus* and *Penicillium* species. Based on valid international standards, a maximum permitted level of patulin is 50 µg L^{-1} in fruit products. In the present study, patulin content of seventy two apple juice samples from West Azerbayjan Province of Iran was determined through High Performance Liquid Chromatography (HPLC). Results revealed the contamination of all the analyzed samples with toxin at a mean content of 48.64 µg L^{-1}. Almost 29% of samples were contaminated with a dose more than 50 µg L^{-1} of patulin. Implementation of appropriate agricultural, as well as, technical practices in apple juice preparation factories of the region is recommended, in order to decrease the contamination level and its deleterious risks.

Keywords: Apple juice, HPLC, Iran, Incidence, Patulin.

INTRODUCTION

Food safety is a global concern due to an increasing awareness of consumers concerning exposure of foods to chemicals and their contamination with such hazardous biochemicals as dioxins, mycotoxins, pesticides, polycyclic aromatic hydrocarbons, drugs and hormones (Barreira *et al.,* 2010). Mycotoxins are the secondary metabolites of filamentous fungi. Despite the various efforts for prevention, mycotoxins remain as a major problem for human health in several parts of world including developed countries. Patulin (4-hydroxy-4H-furo [3, 2c]-pyran-2 [6H]-on) is one of the most injurious mycotoxins (Laidou *et al*., 2001) produced by a wide range of *Aspergillus* and *Penicillium* species (Yuan *et al*., 2010) mainly when the surface tissue of the fruit is damaged. Washing of fruit and removal of spoiled and damaged parts (Leggott *et al*., 2000; Gokmen *et al*., 2001) are low cost procedures to decrease the patulin incidence in the processed fruit. Patulin is mainly found in apples and apple products and occasionally in other such fruits as pears, apricots, peaches and grapes. Although, a lack of mutagenic activity has been reported for this toxin in short-term assays, subchronic exposure may cause teratogenic effects (Kubacki, 1986). Patulin shows various detrimental effects, including DNA damage, neurotoxicity, and development of allergies. As well, the toxin may harm the immune system in humans through long-term consumption of foods and

[1] Food and Hygiene Control Laboratory, Deputy for Food and Drug, Urmia University of Medical Sciences, Urmia, Islamic Republic of Iran.

[*] Corresponding author; e-mail: sh_forouzan2005@yahoo.com

[2] Department of Food Science, Technology and Engineering, University College of Agriculture and Natural Resources, University of Tehran, Karaj, Islamic Republic of Iran.

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beverages contaminated by it (Kawamoto *et al*., 2008).

Many countries have set limits to patulin levels in fruit juices and other fruit products. Codex recommends a maximum permitted level of 50 µg L^{-1} for apple juice (Codex Alimentarius Commission, 2003); whilst, the European Commission (EC) adopted the maximum permitted level of 10 µg kg^{-1} for apple products intended for infants and young children (FAO, 2004). The Joint FAO/WHO Expert Committee on Food Additives (JECFA) estimated the provisional maximum tolerable daily intake (PMTDI) of 0.43 µg kg^{-1} bw day^{-1} or 3.01 µg kg^{-1} bw $week^{-1}$ as safe exposure level (Bolger, 2002). In Iran, the maximum tolerated level of patulin in apple juice is 50 µg L^{-1} (FAO, 2004; ISIRI, 2002). Because of the health concerns regarding patulin and the related ordained regulations, several studies have been conducted to monitor the level of patulin contamination in various countries. Burda (1992) reported that some 23% of fruit product samples surveyed in years 1989 and 1990 in UK were contaminated with patulin; 22% of the samples bearing patulin contents of 51-1,130 µg L^{-1}. From among 100 apple juice samples surveyed for the incidence of patulin in Spain, 82 were contaminated (Prieta, 1994). Another more comprehensive survey on 215 apple juice concentrates, analyzed in Turkey for patulin in 1994, the contamination was demonstrated to stand within the range of 7-376 µg L^{-1} (Gokmen and Acar, 1998). Surprisingly, in Netherlands, only one organically cultivated apple juice was contaminated with patulin (Boonzaaijer *et al*., 2005). In Saudi Arabia, 17 different types of apple juices (51 samples) were analyzed for patulin during 2008. Patulin was detected in only one type with a concentration of 152.5 µg L^{-1} (Al-Hazmi, 2010).

Studies reveal the contamination of a noticeable percentage of apple products with patulin in Iran. Cheraghali *et al*. (2005) reported 33 and 56% of the analyzed apple juices and concentrates contaminated with more than 50 µg L^{-1} of patulin. Another more recent study in northeast region of Iran (Mashhad) indicated that 54 apple juice samples out of total 58 were positive for patulin at levels ranged from 10.5 to 121.8 µg L^{-1}, with 6 samples being contaminated with patulin levels higher than 50 µg L^{-1} (Karimi *et al*., 2008).

West Azerbayjan Province, northwest of Iran, is amongst the largest producers of apple and apple products in the country. Iranian Ministry of Jihad-e-Agriculture (2012) estimated that ~700,000 metric tons of apple were processed in the province during year 2010 contributing to ~30,000 tons of apple juice export abroad including European Union countries. It is therefore required of the governmental bodies to monitor the mycotoxin contamination level in apple products from West Azerbaijan Province of Iran. The present communication reports the analyses results accomplished by Food Control Laboratories during year 2010 supervised by the Ministry of Health as the official body responsible for testing of foodstuffs in Iran.

MATERIALS AND METHODS

Samples

A number of 72 clarified apple juice samples produced in West Azerbaijan Province of Iran during year 2010 were collected randomly by inspectors of Food Control Laboratories of Urmia University of Medical Sciences from different factories. The sampling was performed according to the standard and national procedures (ISIRI, 1987). The samples were cold stored below 4°C before analysis.

Chemicals and Reagents

Patulin (purity≥ 98%) and HydroxyMethyl Furfural (HMF) were procured form Sigma (MO, USA). Ethyl acetate, acetonitrile, ethanol and methanol

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(all HPLC grade), acetic acid and anhydrous sodium carbonate (both extra pure grade), perchloric acid and anhydrous sodium sulphate were purchased from Merck (Darmstadt, Germany). Microfilter membrane (pore size 0.45 µm) was supplied through Millipore (Schwalbach, Germany).

Standard and Working Solutions

Patulin stock solution (200,000 µg L^{-1}) was prepared in ethyl acetate via transferring 5 mg pure crystalline patulin into a 25-mL volumetric flask and dissolving in ethyl acetate. The flask was wrapped tightly with aluminum foil and stored at –20°C. When needed, an appropriate volume of the stock solution was evaporated and then diluted with ethanol to obtain the intermediate standard solution. The solution could be stored at 4°C for 1 month. Concentration of the intermediate standard solution was determined by use of an ultra violet-visible spectrophotometer (Spectrophotometer, Shimadzu, UV 1700, Japan) following the AOAC Official method No. 49.7.02 (AOAC 2000) and ISIRI procedure No. 7438 at 276 nm, against a solvent blank. The final standard solution (1,000 µg L^{-1}) was prepared through evaporating the intermediate standard solution up to dryness under nitrogen stream at room temperature. Immediately, it was supplemented with acetic acid solution (water adjusted to pH 4.0 with reagent grade acetic acid) and mixed well. This solution was diluted with acetic acid in daily preparation of working standard solutions with patulin concentrations of 100, 200, 400 and 500 µg L^{-1}.

Patulin Extraction and Analysis

Patulin was extracted from juice samples following the AOAC official method No. 49.07.02 (AOAC 2000). At first, 10 mL of apple juice was pipetted into a glass culture tube and then 20 mL ethyl acetate added. The tube was then shaken vigorously for 1 minute to extract the patulin into ethyl acetate. The layers were let separated and the upper organic layer transferred to another glass culture tube. A second 20 mL portion ethyl acetate was added again to the first tube and re-shaken. The separated organic layer was combined with its former counterpart and the procedure (adding 20 mL ethyl acetate and extraction) was repeated for the third time. These were followed by adding 4 mL 1.5% Na_2CO_3 solution to the combined ethyl acetate extracts and mixing the tube's contents vigorously. Layers were let separated after which, the upper ethyl acetate layer was transferred to a clean tube, and the lower aqueous layer further extracted with fresh 10 mL of ethyl acetate. The obtained ethyl acetate layer from the aqueous phase extraction was mixed with the first extract and the lower aqueous layer discarded. The extract was passed through 15 gr Na_2SO_4 followed by vigorous shaking of filtrate for 30 seconds and heating at 40ºC under nitrogen stream. The dry remainder was dissolved in 1 mL acetic acid for High Performance Liquid Chromatography (HPLC) analysis.

HPLC analysis was performed applying a reverse-phase HPLC system (HPLC, Waters, USA) equipped with a vacuum degasser (in-Line degasser, AF waters), pump (Binary HPLC pump, Waters, model 1525) temperature-controlled oven, auto sampler (Waters, 717 plus) and an ultra-violet detector (dual λ absorbance detector, Waters, model 2487, USA) set on 276 nm. A stainless steel analytical column (250×4.6 mm i.d., 4 µm, Synergy Hydro-RP C_{18}) preceded by a guard column (4×3 mm i.d.) with the same stationary phase was made use of. The isocratic mobile phase was water-acetonitrile at a ratio of 93:7 with a flow rate of 1 mL min^{-1}. Data collection and subsequent processings were performed using the Breeze software (Breeze, version 3.30, USA).

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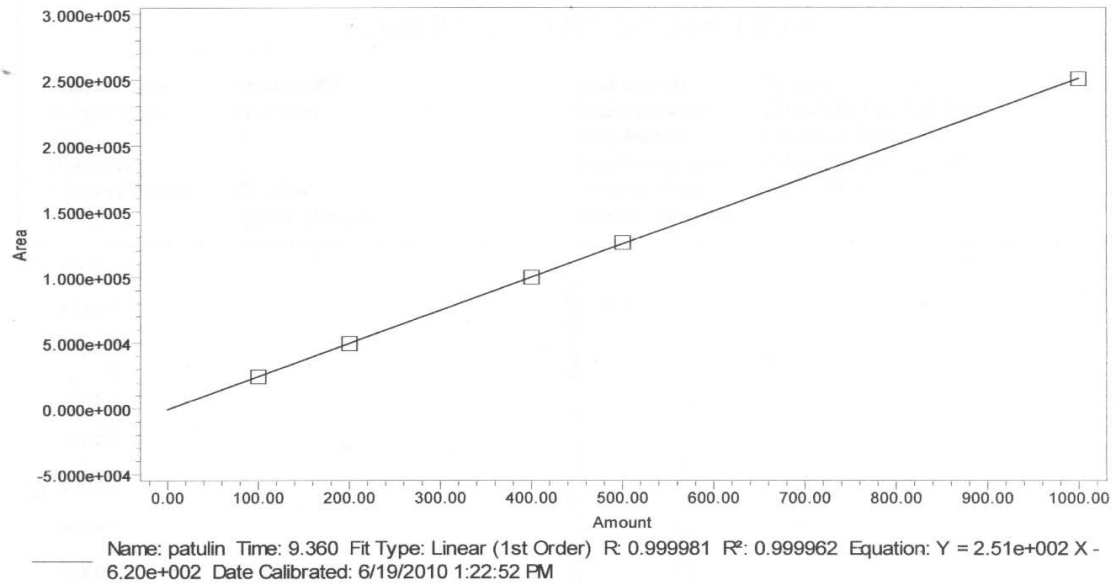
Calibration Curve

A calibration curve was prepared by using the patulin working solutions with various concentrations of 100, 200, 400, 500 and 1,000 µg L^{-1}. The curve (Figure 1) was constructed before the analysis to check the pilot for linearity (r^2= 0.9999) and was used for quantification of patulin. If the content of toxin in the sample was outside the calibration range, a more appropriate calibration curve was prepared, or the injection solution for HPLC analysis was diluted to a patulin concentration appropriate for the established calibration curve. The detection limit (LOD) of the method was determined through the successive analyses of chromatographic extracts of apple juice concentrate samples spiked with the decreasing amounts of patulin until a signal to noise ratio of 3:1 was reached. To determine the retention times of patulin and HMF, a mixed standard (500 µg L^{-1} patulin+500 µg L^{-1} HMF solutions) was injected to HPLC apparatus.

Quality Assurance

For an evaluation of the reliability of results, in addition to using validated methods, internal quality control experiments were performed. Along this line, recoveries of patulin were recorded by analyzing an apple juice sample spiked at 50 µg L^{-1} of patulin. Figure 2 demonstrates the chromatogram for apple juice spiked with 50 µg L^{-1} patulin, as well as, that for a naturally contaminated juice sample. According to the recovery values, patulin levels were corrected for recoveries. The recovery of patulin obtained by spiking the apple juice with 50 µg L^{-1} was 86%.



	Sample name	Peak name	Level	Amount	Response	Calc. amount	% deviation	Manual point	Ignore point
1	Patulin 100 ppb	Patulin	1	100.000	2.432e+004	99.201806	-0.798	No	No
2	Patulin 200 ppb	Patulin	2	200.000	4.938e+004	198.889955	-0.555	No	No
3	Patulin 400 ppb	Patulin	3	400.000	9.981e+004	399.488668	-0.128	No	No
4	Patulin 500 ppb	Patulin	4	500.000	1.260e+005	503.826419	-0.765	No	No
5	Patulin 1000 ppb	Patulin	5	1000.000	2.504e+005	998.593152	-0.141	No	No

Figure 1. Standard curve for patulin.

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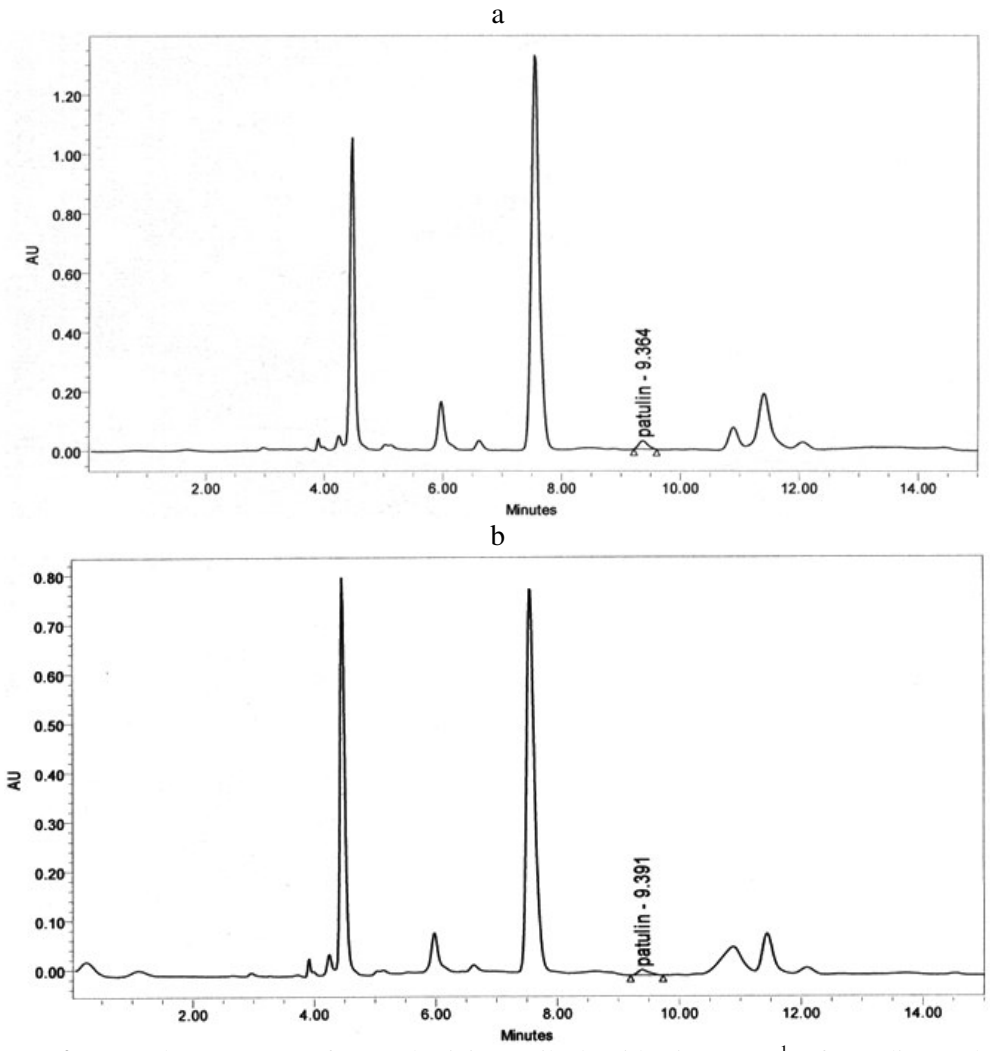

Figure 2. (a) Chromatogram for apple juice spiked with 50 µg L^{-1} of patulin, and (b) Chromatogram for a contaminated apple juice.

RESULTS

Method's performance, determined through the internal quality validation, showed that LOD was 5 µg L^{-1} indicating a good performance at relatively low statutory limits. As well, the Limit Of Quantification (LOQ) was acceptable (15 µg L^{-1}) implying the reliability of the generated data. Cunha *et al*. (2009) validated a gas chromatographic mass spectrometric method for the determination of trace levels of patulin in apple products and quince jam with LOD of 0.4 µg L^{-1}. The Relative Standard Deviation for reproducibility (RSD_R) of the analytical method applied in the present study for measurement of patulin in apple juice was 9% showing the reproducibility of results. The main interfering compound in patulin determination is HMF which is formed due to either heat treating of carbohydrates present in apple juice or during long-term storage (Karimi *et al*., 2008). It is therefore required to obtain a chromatogram with well separated baselines for these two compounds in patulin measurement. Figure 3 shows the well separated chromatogram of patulin and

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HMF obtained before toxin measurement in juice samples.

Table 1 reports the results for incidence of patulin in apple juice produced in factories of West Azerbayjan Province. All the analyzed samples were contaminated with patulin at concentrations ranging from 29.58 to 151.2 µg L^{-1}.

DISCUSSION

Results obtained in the present study indicate the widespread contamination of apple juice produced in northwest of Iran with mycotoxin patulin albeit not at high levels. All the samples analyzed were proved toxin-positive due most probably to the processing of improperly harvested and physically injured apples used in juice extraction. Fruits of appropriate ripeness, quality and appearance are traditionally used up as fresh in Iran whilst, sub-tree and injured ones processed to juice. As well, fruits are usually stored for prolonged time intervals under sunlight in orchards, unlimbering the growth and propagation of toxin-producing organisms. It is also worth notice that post-harvest transportation and delivery of fruits to juice plants is still not well mechanized contributing to the loss and injury of the produce. Barreira (2010) studies in Portugal indicated that patulin was detected in solely 23% of apple-based food samples with values ranging from 1.2 to 42 µg kg^{-1}. As well, the toxin even though not detected in infant drinks, but it was found in 7% of homogenized apple puree samples intended for infants' and young children's consumption. Barreira detected a higher incidence of toxin in cloudy juices.

Despite the vast incidence of patulin in samples analyzed within the present study, the mean contamination level was promisingly lower than the maximum permitted level by EU and the Codex i.e. 50 µg L^{-1}. The toxin in ~29% of samples was quantified at concentrations more than 50 µg L^{-1} which is in close match with that already reported for samples from Iran by Cheraghali *et al*. (2005). However, the study carried out in northeast of Iran showed that

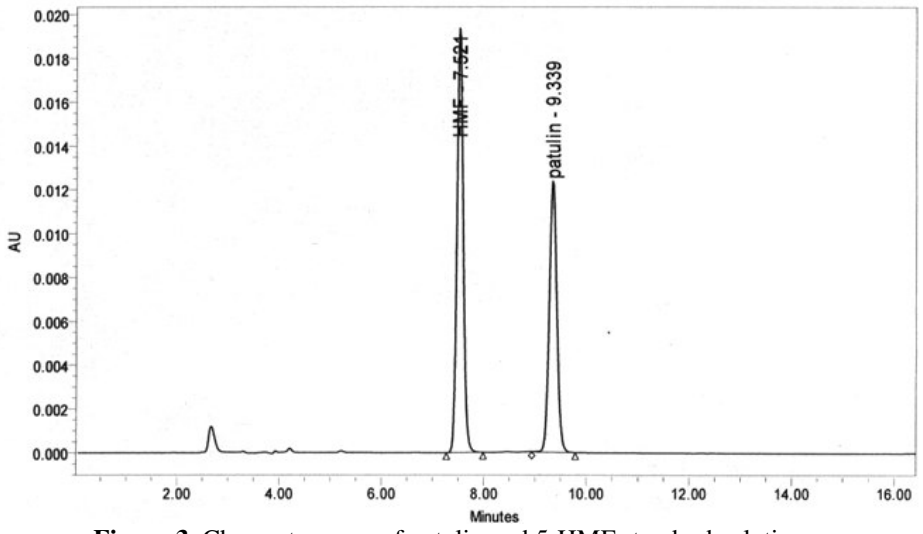

Figure 3. Chromatograms of patulin and 5-HMF standard solutions.

Table 1. Patulin content in apple juices from Urmia.

Total Number	Number (%) of samples containing patulin				Mean ± SD
	< 15 µg L^{-1}[a]	15-50 µg L^{-1}	> 50 µg L^{-1}	Max (µg L^{-1})	
72	8 (11.1%)	43 (59.7%)	21 (29.16%)	151.2	48.64±4.4

[a] 15= Limit of quantification.

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~10% of apple juice samples had patulin levels higher than 50 µg L^{-1} (Karimi *et al*., 2008). In a survey on occurrence of patulin in commercial pure apple juices (53 samples) and mixed apple juices (82 samples) marketed in Italy, the toxin was quantified in 34.8% of samples within the range of 1.58–55.41 µg kg^{-1}. With the exception of one sample, the level of patulin was lower than 50 µg kg^{-1} (Spadaro *et al*., 2007). Results of a study done by Yuan (2010) in China showed that patulin was present in 16% of apple products at levels higher than 50 µg L^{-1}. Murillo-Arbizo (2009) reported that, in 11% of the apple juice samples analyzed in Spain, patulin contamination exceeded the maximum permitted level of 50 µg L^{-1}. Gokmen (2000) studies on 482 apple juice concentrates produced through 1996–1999 showed that year to year variations in patulin levels of apple juice concentrates were found out as statistically significant. Patulin contamination levels of apple juice concentrates tended to decrease through the years. In a study on patulin contamination in apple juice (12 samples) sold in retail outlets in Italy and South Africa, half of the samples showed patulin contamination, of which four had levels well above the acceptable limits (Katerere *et al*., 2007). The results of the study on incidence of patulin in Brazilian apple-based drinks showed that this toxin does not seem to be a problem in apple-based drink commercialized in the State of Sao Paulo (Helena Iha and Sabino, 2008).

The maximum patulin content measured in the present study (Table 1) was significantly lower than that reported by Burda in 1992 for Australian apple juice concentrate namely 646 µg L^{-1}. As well, a minimum contamination level of 150 µg L^{-1} was found in Brazil for 14 apple samples (De Sylos and Rodriguez-Amaya, 1999). Aktaş *et al*. (2004) analyzed the patulin level of apple juices in the Isparta region of Turkey and found that expect for one group, the toxin level did not exceed 50 µg L^{-1}. The mean patulin content in apple juice samples surveyed in the present study (Table 1) was higher than that reported by Karimi and others (2008) for apple juice from Mashhad (northeast of Iran) which was 29.2 µg L^{-1}. As well, the population of samples contaminated with > 50 µg L^{-1} toxin was higher than that reported by those researchers. This is probably due to problems in fruit washing, pulping, heat treatment and storage processes in West Azerbayjan factories. It is also worth attention to note that variety of apple may influence the potential of fruit for contamination by mycotoxins. Varieties Red Delicacies and Golden Delicious that are majorly harvested in West Azerbayjan Province are sweet taste, whilst, the local variety Abbasi in northeast of Iran is more acidic. A high acidity may suppress the molds and restrict their growth (López-Malo, 2005) leading to a lower secretion of toxin. The patulin contamination of apple products can be controlled in any or all of the three manners of: prevention of patulin contamination in the harvesting, processing, and storage steps; removal of patulin during processing; and/or post production treatments to remove or detoxify the toxin.

CONCLUSIONS

The mean concentration of patulin in apple juice samples produced in the northwest of Iran was slightly lower than the maximum level permitted by Codex Alimentarious. However, the high incidence of mycotoxin in apple juices indicates the need for alertness of the apple industry to patulin contamination. It is highly demanded to implement the improved techniques for production of apple products in order to reduce the incidence and level of patulin contamination in apple juices and in the concentrates. It is worth notice that consumption of apple juice products produced in the northwest of Iran is harmful to children as the maximum level allowed for apple products intended for infants' and young children's consumption is 10 µg kg^{-1} (EC, 2003). Proper apple selection,

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handling, sorting, storage, reception, and washing can assure that only good quality, uncontaminated fruit is utilized in being changed into sound and safe apple products. The use of these proper manufacturing practices can assure the substantial and effective reduction of patulin (Janotova *et al*., 2011). It is finally proposed to apply similar enforcement and even stricter control measures to manage the control of contamination (by promoting acceptable agricultural practices in the orchards, hazard analysis as well as implementation of critical control point principles in storage and processing plants) as is alreadycarried out in the management and processing of pistachio product (Dini *et al*., 2012).

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میزان شیوع پاتولین در نمونه‌های آب سیب تولید شده در استان آذربایجان غربی

ش. فروزان، ا. مددلو

چکیده

به دلیل افزایش آگاهی مصرف‌کنندگان در مورد آلودگی مواد غذایی به مواد شیمیایی و بیوشیمیایی خطرناک مانند دی‌اکسین، مایکوتوکسین‌ها، آفت‌کش‌ها، هیدروکربن‌های آروماتیک چند حلقه‌ای، داروها و هورمون‌ها، ایمنی مواد غذایی به یک نگرانی جهانی تبدیل شده‌است. پاتولین یکی از مهم‌ترین مایکوتوکسین‌های تولید شده توسط انواع قارچ‌ها، به ویژه گونه‌های آسپرژیلوس و پنی‌سیلیوم است. بر اساس استانداردهای معتبر بین‌المللی، حداکثر مقدار مجاز پاتولین در میوه‌جات، ۵۰ میکروگرم بر لیتر می‌باشد. در تحقیق حاضر، مقدار پاتولین ۷۲ نمونه آب سیب تولیدشده در کارخانجات استان آذربایجان غربی، توسط کروماتوگرافی مایع با کارایی بالا اندازه‌گیری شد. نتایج حاکی از آلودگی تمام نمونه‌های مورد تجزیه با سم پاتولین با میانگین ۴۸/۶۴ میکروگرم بر لیتر بود. تقریبا ۲۹ درصد نمونه‌ها با مقدار بیش از ۵۰ میکروگرم بر لیتر آلوده به پاتولین بودند. نهایتاً اجرای شیوه های مناسب تولید کشاورزی در کارخانجات تولید آب سیب به منظور کاهش سطح آلودگی و خطرات، توصیه می شود.

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