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Determination of Patulin in Clear Apple Juice using High Performance Liquid Chromatography with Diode Array Detection

A.M. Gomaa; M.E. Amer and E.R. Attallah

Central Lab. of Residue Analysis of Pesticides and Heavy Metals in Foods, Agric. Res. Centre, Ministry of Agric. and Land Reclamation.

Patulin is biosynthesized toxin produced by several species of filamentous fungi such as *Penicillium* spp., *Aspergillus* spp. and *Byssochlamys nivea*. The moulds grow on a variety of foods including fruits and fruit products.

Patulin was recovered at different levels varied between 70-120%. The reproducibility expressed as relative standard deviation was less than 15%. The limit of quantification was 15µg/l. The method showed to be linear from the limit of quantification up to 200µg/l. The method accuracy was tested by participating in FAPAS proficiency test program. Forty-eight samples of clear apple juice collected from Egyptian local markets, half of these samples were from local production and the other imported from different countries. Patulin was detected in 22 out of 48 analyzed samples, at concentration levels ranged from 6.68 to 785.0 µg/l. The amount of patulin detected in 12 analyzed samples showed exceeding of permissible limit.

Keywords: Apple juice, *Aspergillus* spp., *Byssochlamys nivea*, mycotoxins, patulin and *Penicillium* spp.

Patulin (4-hydroxy-4H-furo-[3, 2-c] pyran-2(6H)-one) is a secondary metabolite produced by some species of *Aspergillus, Byssochlamys* and *Penicillium* (Weidenborner, 2001). Apple and its products are the proper substrates for *Penicillium expansum*, the causal agent of blue mould, to produce the mycotoxin. The fruit pathogen is generally associated with damaged fruit or fruit already infected by other microorganisms in orchard as well as at post harvest conditions (Snowdon, 2001).

Symptoms in experimental cases of patulin toxicosis in animals are lung and brain edema, liver, spleen and kidney damage and toxicity to the immune system (Llewelly *et al.*, 1998). For humans, nausea, gastrointestinal disturbances and vomiting have been reported (Lai *et al.*, 2000).

The World Health Organization (1995) and CODEX alimentations recommend a maximum permitted level of 50 µg/l for apple juice. Recently, for patulin, the following allowed levels were established by European Commission (EC): 50 µg/kg for fruit (apple) juices and apple juice ingredients in other beverages; spirit drinks, ciders and other fermented drinks derived from apples; 25 µg/kg for solid products including apple compote, apple puree; 10 µg/kg for apple products intended for infants and young children (Jian-ke *et al.*, 2007).

Many methods have been developed for measuring patulin in apple juice these include methods based on thin-layer chromatography (TLC), gas chromatography (GC) and more recently high-performance liquid chromatography (HPLC) (Trucksess and Tang, 1999; Boonzaaijer *et al.*, 2005; Vural *et al.*, 2005 and Jian-ke *et al.*, 2007). Conventional methods for the determination of patulin are based on liquid–liquid extraction with ethyl acetate as described by some authors (McDonald and Illida, 1997 and McDonald *et al.*, 2000).

In the present study, a simple and reliable method for determination of Patulin in clear apple juice using high-performance liquid chromatography with diode array for detection is described. The method was validated and measurement uncertainty was estimated according to Eurachem and Eurolab guidelines (Anonymous, 1998; Anonymous, 2000 and Eurolab, 2007) the validated method was applied to determine the levels of Patulin in samples collected from Egyptian local markets including local and imported products.

Materials and Methods

Sampling:

Forty-eight manufactured samples of clear apple juice collected from Egyptian markets, 24 samples distance produced locally and the others distance imported from different countries. Two packages of each sample with same patch number and within the valid expire date were collected. The samples were kept in refrigerator at 4°C until analysis. One sample subjected for analysis and the other was kept as reference.

Apparatus:

High performance liquid chromatography (HPLC), HP 1100 series equipped with: Quaternary pump, Vacuum degasser, Auto sampler and Diode Array Detector (DAD). Analytical Column: C18, 5µm, 25cm, 4.6mm. Rotary evaporator (Heidolph VV2000). UV- Spectrophotometer (UNICAM).

Reagents and chemicals:

All solvents are of HPLC grade, Acetonitrile, Chloroform, Ethyl Acetate, Acetic acid, glacial ≥ 99.5%, Ethanol ≥ 99.7% AR quality. ≥ 99%., anhydrous sodium carbonate, anhydrous sodium sulfate, De-ionized water, Sodium carbonate solution 1.5%: Acidified water, pH =4. 5-Hydroxymethyl furfural (HMF) and patulin standard were purchased from Sigma.

Reference standard solutions:

a) Stock solution:

Patulin is prepared by dissolving 10 mg of Patulin in 5 ml chloroform. Stock solution was kept in a liquid nitrogen tank at (-18°C).

b) Intermediate solution:

A volume of 10 µg /ml of Patulin is prepared by transferring appropriate volume of the stock solution into volumetric flask and evaporated just to dryness under a gentle stream of nitrogen at room temperature. The residue was immediately dissolved in ethanol. The exact concentration of the intermediate solution was determined by spectrophotometry measurements according to the AOAC (2002); Intermediate solution was kept in a liquid nitrogen tank at (-18 to -23°C).

c) Working and calibration solution:

2 ug/ml of Patulin solution is prepared by pipitting appropriate volume of intermediate solutions into volumetric flask and evaporating just to dryness under a gentle stream of nitrogen at room temperature. The residue was immediately dissolved in acidified water and kept in freezer at (-18°C). Portion used for routine work was kept at 4°C for maximum two weeks.

Extraction:

The followed method is based on the method of AOAC (2002). A modification has been done to overcome the interfering compounds initiated from extraction step and replaced perchloric acid by acetic acid in HPLC mobile phase. The current study describes, in details, the procedure after modification. Ten ml of clear apple juice sample were transferred into a 100 ml separating funnel. 20 ml of ethyl acetate was added and shake for one minute. The layers allowed to be separate then drain them into two separate conical flasks. The aqueous layers transferred back into the same separating funnel and re-extracted with a second 20 ml portion of ethyl acetate. The lower aqueous layer drained into clean conical flask and the upper layer into another conical flask containing the ethyl acetate layer from the first extraction. The extraction procedure was repeated three times. The aqueous layer was discarded and the three portions of ethyl acetate were combined in the separating funnel. The conical flask used to collect the ethyl acetate phases was rinsed with a further 5 ml ethyl acetate and added to the ethyl acetate extract in the separating funnel.

Cleanup procedure:

Removal of interfering acidic compounds:

Four ml of sodium carbonate solution were added to the separating funnel from previous step and shake for 0.5 minute. The layers were allowed to separate. The lower aqueous layer poured was drained into a conical flask. The upper layer was transferred into a round-bottomed flask through a funnel and cotton containing 15 g anhydrous sodium sulfate. The aqueous layer was transferred back into the separating funnel. The conical flask was rinsed with 10 ml ethyl acetate, and added to the separating funnel then shake for 0.5 minute. The layers were allowed to separate. The lower layer was discarded, and the upper layer filtered through anhydrous sodium sulfate into the round-bottomed flask. The sodium sulfate was washed twice with 10 ml of ethyl acetate and collected in the round-bottomed flask then evaporated using rotary evaporator at 40°C till near dryness. About 5 ml from the solvent were left otherwise for the complete evaporation till dryness to reduce the recovery dramatically, then immediately redissolve in a final volume of 1 ml of acidified water and transferred to an HPLC vial. If necessary, the sample may be filtered before analysis by using Acrodisk of membrane size 0.45 µm.

Check with Acrodisk was made using standard solution to assess any loss of patulin before the filtration of tested extracts.

Quantification condition of HPLC–DAD:

Flow rate: 1 ml/min., injection volume: 20 µl. Diode array detector set at 276nm. Mobile phase: 1% acetonitrile (in acidified water pH= 4). The gradient started at the concentration of 1% acetonitrile solution and ending at 99% acetonitrile after 35 min as shown in (Table 1).

Table 1. The elution program for separation

Time/min	Acetonitrile (%)	Acidified water (%) pH=4	Flow Rate ml/min
0.00	0.00	100	1.0
5.00	1.00	99	
22.00	5.00	95	
22.10	100	0.00	
25.00	100	0.00	
25.10	1.00	99	

Optimization of HPLC conditions:

Comparison of the AOAC (2002) method and the modified method has been done. The use of gradient mobile phase started with 1% Acetonitrile and 99% acidified water pH= 4 at fixed flaw rate 1 ml /min improved the separation and overcome interference problem of HMF and other compounds.

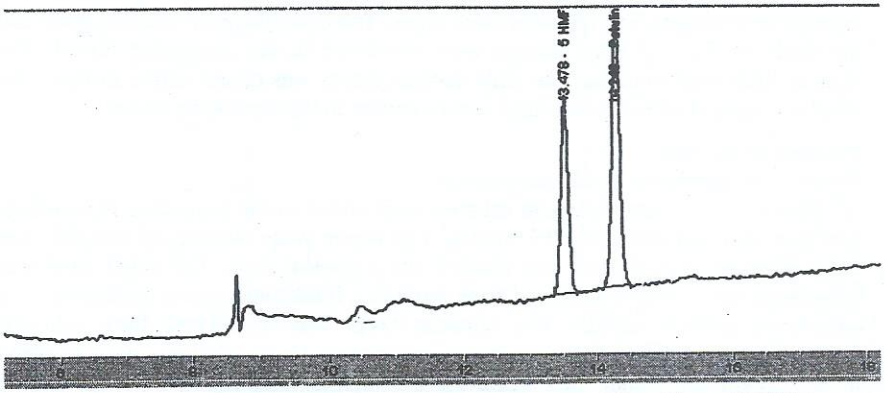

Fig. 1. Chromatogram of 250ng/ml standard solution of Patulin and HMF.

Method validation:

The parameters of Eurachem guidelines (Anonymous, 1998) were selected for the validation of the adopted method as follows:

Limit of Detection (LD):

The limit of detection is estimated as 3s (s= Standard division) of sample blanks fortified at lowest acceptable concentration level (LOQ). The limit of detection (LD) for Patulin was 4.5 µg/l.

Limit of Quantification (LOQ):

The lowest practical limit of quantification was estimated by using repeated spiked samples at about the expected lowest quantification level that is 15 µg /l, on clear apple juice samples. The recovery and precision data of LOQ are shown in Table (2)

Recovery tests:

The recovery tests for Patulin were performed by using repeated spike samples on clear apple juice at different concentration levels. The average recoveries and relative standard deviation on each level were calculated (Table 2).

Table 2. Results of recoveries percentages of Patulin at different concentration levels on apple juice samples

Spiking level (µg/l)	Average recoveries (%)	CV (%)*
15	76	13
25	81	13
50	85	10
100	86	7
200	88	2

* CV= coefficient of variance.
Six replicates were used for each level.

Linear range:

For quantitative analysis, the range of analyte concentrations over which the method may apply, was determined by injection of five different concentration levels 15, 25, 50, 100 and 200 µg/ml. The correlation coefficient is greater than 0.999.

Method Accuracy:

The method trueness was confirmed by participation in 2 proficiency testing (PT) programs organized by FAPAS, CSL-UK. Table (3) show accepted z-scores for these rounds.

Table 3. Proficiency testing results on clear apple juice samples

Round No.	Assigned value µg/l	Lab results µg/l	z-score
1624	39.6	30.0	-1.1
1632	37.5	24.5	-1.6

Satisfactory z-score was between $-2 \leq z \leq 2$

Measurement Uncertainty:

Measurement uncertainty is estimated using validation data according to Eurachem guidelines (Anonymous, 2000). Different components are contributing in the total uncertainty.

Precision:

The random effects were estimated as the relative standard deviation of spike samples during two years, 7/2006-7/2007. Relative standard uncertainty due to precision experiments (Up), expressed as relative standard deviation was found to be 6.0%.

Bias:

The bias of the analytical procedure was investigated during the long term study using spiked samples. The mean recovery (86%) was observed with standard deviation s= 8.7% and n= 25. The standard uncertainty was calculated as the standard deviation of the mean is 1.7%.

A significance test was applied to test if the recovery is significantly different from 100 %. For 24 degrees of freedom t_{tab} = 2.06 and t_{calc}=8.03. In this case (since t_{calc} is greater than t_{tab}) the recovery is statistically significantly different from 100%, but in the normal application of the method no correction is applied. The uncertainty increased (Ui) to take account of the fact that the recovery has not been corrected for. The relative standard uncertainty due to bias (U_{bias})= 8.4%.

Other uncertainty sources:

All balances and the important volumetric measuring devices are under regular control. Precision and recovery studies take into account the influence of the calibration of the different volumetric measuring devices because during the investigation various volumetric flasks and pipettes have been used. The uncertainty due to reference standard preparation was estimated by accounting for reference standard purity tolerance, balance, volumetric flask and pipettes. The uncertainty component due to reference standard preparation was very small and could be neglected (<1 %).

Combined Uncertainty (U_C):

Combined uncertainty, is the positive square root of the sum of the squares of different uncertainty components, was found to be less than 10.3%. The following equation was used for combined uncertainty calculations:

$$U_C = \sqrt{(U_p)^2 + (U_{Bias})^2}$$

Expanded Uncertainty:

Expanded uncertainty is obtained by multiplying the combined uncertainty, by a coverage factor k, for confidence level of 95% k is 2. The expanded uncertainty (at 95% confidence level) was found to be ±21%.

Results and Discussion

Recent surveys on patulin occurrence concerned apples, clear apple juices, or apple nectar were reported (Beretta *et al.*, 2000; Tangni *et al.*, 2003 and Baert *et al.*, 2006). In a previous study, higher patulin concentrations were found in organic apple juice (8.8 µg/l) compared to conventional (4.1 µg/l) and handcrafted apple juice (4.4 µg/l) (Snowdon, 2001).

The validated method was used in the surveillance study of patulin levels in clear apple juice samples collected from Egyptian local markets during 2006 and 2007 including the imported and the locally produced packages.

Table (4) and Fig. (2) show the results of patulin levels in µg/l in the collected samples. Seventeen samples of locally produced clear apple juice 100% were analyzed for patulin. The results showed that, 7 samples were contaminated with patulin with a percentage of 41.2 %. The contamination levels ranged between 6.86 and 749.0 µg/l. Three samples out of the contaminated samples contain levels of patulin exceeding the codex and EU maximum permissible level (50 µg/l) with a mean of 84.5 µg/l. In case of nectar 50%, 6 of 7 samples were contaminated with a percentage of 85.7% and 4 samples (57%) had more than 50 µg/l. The contamination levels ranged between 16.8 and 689.0 µg/l with a mean of 140.5 µg/l.

The study was also included analysis of patulin in 21 imported clear apple juice 100% samples; 9 samples were contaminated with patulin with a percentage of 42.9 %.The contaminated levels ranged between 31.0 and 785 µg/l with a mean of 119.6 µg/l. The results showed also that all samples of apple juice contain carbonate are free of patulin

Table 4. Patulin levels in clear apple juice containing 100% apple juice or certain percentage of apple marketing in Egypt

Source of samples	Type of apple juice samples	Total No of samples	No of contaminated samples	No of samples <15 µg/l	No of samples 15-50 µg/l	No of samples >50 µg/l*	Mean µg/l	Min µg/l	Max µg/l
Local products	Apple juice 100%	17	7	3	1	3(17.6%)	84.5	6.86	749
	Nectar 50 %	7	6	-	2	4(57%)	140.5	16.8	689
Imported products from different countries	Apple juice 100%	21	9	-	4	5(23.8%)	119.6	31.0	785
	Carbonated apple juice	3	-	-	-	-	-	-	-

* The number of samples exceeding the permissible limit 50 µg/l.

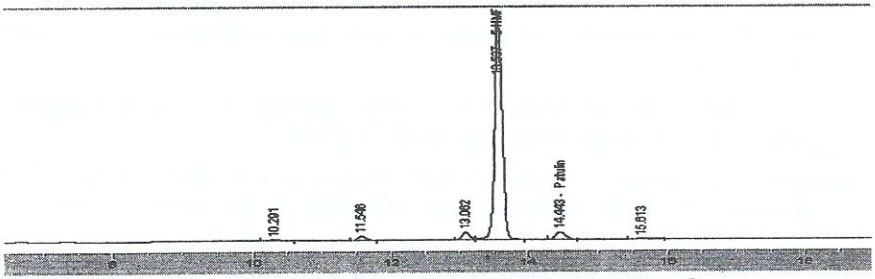

Fig. 2. Chromatograms of contaminated clear apple juice sample.

Conclusion

The validated method showed satisfactory recoveries and precision. The method accuracy was confirmed by satisfactory results of proficiency testing samples. The described method could be used in routine inspection of patulin in clear apple juice samples according to EU maximum permissible limits.

The levels detected in some samples (17-57% violation percentages) from both local produced and imported samples impose extensive obligatory control of the consumed apple juice throughout national monitoring program

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تقدير الباتيولين فى عصير التفاح باستخدام جهاز الكروماتوجرافى السائل عالى الكفاءة والملحق بكاشف متعدد الاطياف

أحمد ممدوح جمعة ، محمد السيد عامر، عماد رمضان
المعمل المركزى لتحليل متبقيات المبيدات و العناصر الثقيلة فى الأغذية ،
مركز البحوث الزراعية ، وزارة الزراعة واستصلاح الأراضى .

الباتيولين من السموم الفطرية المخلقة بيولوجيا بواسطة العديد من سلالات الفطريات الخيطية مثل جنسى *Penicillium* و*Aspergillus* والفطر *Byssochlamys nivea* . وتنمو هذه الفطريات على العديد من الأغذية والتى تشمل الفواكة و المنتجات المشتقة منها ويتم استخلاص الباتيولين بواسطة استر أسيتات الإيثيل، وينقى المستخلص من الشوائب باستخدام كربونات الصوديوم والذي يقدر كميا باستخدام جهاز السائل الكروماتوجرافى عالى الكفاءة والملحق بكاشف متعدد الأطياف . يتراوح متوسط الاسترجاع للباتيولين ما بين ٧٠ الى ١٢٠% عند مستويات مختلفة. وتم التعبير عن معدل الانتشار باستخدام الانحراف المعيارى النسبي وكانت قيمته أقل من ١٥% ، كانت حدود التقدير ١٥ ميكروجرام/ لتر . كما أن طريقة التحليل تبدى تناسبا طرديا بين الاستجابة و التركيز بدءا من قيمة حدود التقدير والتى كانت ١٥ ميكروجرام/ لترحتى ٢٠٠ ميكروجرام /لتر . كما تم اختبار دقة طريقة التحليل بالمشاركة فى برنامج إختبار الكفاءه FAPAS بانجلترا وتم جمع ثمانية و اربعون عينة عصير تفاح من السوق المحلية المصرية حيث كانت نصف هذه العينات محلية الإنتاج والنصف الآخر مستورد من دول مختلفة، ووجد الباتيولين فى ٢٢ عينة من مجمل ٤٨ عينة تم تحليلها بتركيزات تتراوح ما بين ٦,٨٦ الى ٧٨٥ ميكروجرام/ لتر. وكانت كميات الباتيولين المقدرة فى ١٢ عينة من مجمل العينات التى تم تحليلها متجاوزة للحدود المسموح بها. مما يستلزم إدراج تحليل الباتيولين ضمن التحاليل المتعلقة بسلامة الغذاء.