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Analytical Methods

Determination of patulin in fruit juices using HPLC-DAD and GC-MSD techniques

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Abstract

A high performance liquid chromatography with a diode-array detector (HPLC-DAD) and a gas chromatography with a mass spectrometer (GC-MSD) are described for the determination of patulin (PAT) in apple juice. The limits of detection (DL) and quantification (QL) for the HPLC-DAD and GC-MSD method were found to be (DL = 0.23 μg kg^{-1} QL = 1.2 μg kg^{-1}) and (DL = 5.8 μg kg^{-1} and QL = 13.8 μg kg^{-1}), respectively. The recovery factors for HPLC-DAD and GC-MSD were found to be 99.5% (RSD% = 0.73) and 41% (RSD% = 10.03), respectively. The HPLC-DAD method was used to determine the occurrence of PAT in 90 samples of fruit juices.

Results revealed the presence of PAT in 100% of the samples examined. The mean values of PAT in concentrated fruit juices and in the commercial fruit juices collected from the Greek market were found to be 10.54 μg PAT kg^{-1} and 5.57 μg PAT kg^{-1} juice, respectively. The most contaminated samples were four concentrated juices ranging from 18.10 μg PAT kg^{-1} to 36.8 μg PAT kg^{-1} juice. The daily exposure to patulin for the consumers of all ages in Greece, is ranging from 0.008 μg PAT kg^{-1} bw to 0.1 μg PAT kg^{-1} bw if the daily intake of fruit juices is from 0.1 to 0.5 kg. With the exception to the most contaminated sample, the daily exposure due to the samples examined, is below the provisional maximum tolerable daily intake for PAT (0.4 μg PAT kg^{-1} bw).

Keywords: HPLC-DAD; GC-MSD; Patulin; Fruit juices; Risk assessment

1. Introduction

Patulin (PAT) is a mycotoxin produced by *Penicillium (P.), Aspergillus (A.)* and *Byssochlamys* species. Among these fungi *P. expansum* is the most important producer of the mycotoxin which can be detected in fruits and juices (Paster, Huppert & Barkai-Golan, 1995; Sommer, Buchanan & Fortlage, 1974) *P. expansum* is developed often on the surface of healthy fruit. Nevertheless is normally associated with damaged fruits infected by microorganisms in post harvest conditions (Ritieni, 2003).

PAT has been evaluated in apples and their products and sometimes in cereals, bread, pears, apricots, peaches, grapes and products derived from these products (Prieta et al., 1993). According to Marin, Morales, Hasan, Ramos and Sanchis (2006), low quality apples from storage rooms (either under controlled atmosphere (CA) or not) are also used for apple juice production resulting to highly contaminated juices with PAT.

Although PAT was studied firstly as a potential new antibiotic, acute symptoms from PAT consumption can include agitation, convulsions, oedema, ulceration and vomiting (Speijers, 2004). Chronic health effects of PAT include genotoxicity, immunotoxicity and neurotoxicity in rodents while its effects on humans are not clear yet (Wouters & Speijers, 1996). Furthermore, PAT has been shown to be teratogenic (Frayssinet, 1984) but it is classified by the International

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Agency for Research on Cancer in category three as a not classifiable toxic compound regarding its carcinogenicity to humans (IARC, 1993).

Many methods have been developed for measuring patulin in apple juice such as T.L.C. (Harwig, Chen, Kennedy & Scott, 1973), mass spectrometry (Sheu & Shyu, 1999), colorimetry (Subramanian, 1982), gas chromatography/mass spectrometry (Rupp & Turnipseed, 2000). At the moment high performance liquid chromatography with ultra violet light detection (HPLC-UV) is the most frequently used method (Baert et al., 2007; Brause, Trucksess, Frederick & Page, 1996; McDonald, Long & Gilbert, 2000).

The occurrence of PAT in apple juices has been reported in various countries (Cheraghali et al., 2005; De Sylos & Rodriguez-Amaya, 1999; Gökmen & Acar, 2000; Ito et al., 2004; Piemontese, Solfrizzo & Visconti, 2005; Spadaro, Ciavorella, Frati, Garibaldi & Gullino, 2006) . On the contrary, no information exists about the occurrence of patulin in apple juices in the Greek market.

The objectives of the present study were (1) to develop, evaluate and compare two different methods for the determination of patulin in apple juice and (2) to determine the occurrence of PAT in 90 samples of fruit juices either imported or collected from the Greek market. The methods which were used were HPLC with a diode-array detector (DAD) and gas chromatography (GC.) with a mass spectrometry detector (MSD).

2. Materials and methods

2.1. Samples

Ninety samples of fruit juices were collected by the official authorities from October 2004 to June 2006. The samples weighting from 100 g to 1 kg were analyzed at the General Chemical State Laboratory of Greece according to the directive 78/2003 of the European Union. Forty samples were concentrated fruit juices. Among them 23 apple juices were imported from Turkey, 14 apple (Brix number = 11.2) and two apricot juices (Brix number = 11.2) were imported from China. One pineapple juice (Brix number = 12.8) of unknown origin was purchased from a company producing juices. Before analysis the concentrated juices were measured for their Brix number and according to this measurement they were reconstituted as recommended by the association of the industry of juices and nectars (AIJN). In addition forty seven commercial juices ready for consumption were collected from the Greek markets. Among them 29 were apple, three were orange, 12 were mixed fruits juices and one rosehip juice. One sample of apple soft drink and two samples of baby foods containing apple pulp were examined as well.

Each sample was well homogenized (concentrated juices and pulps) or stirred and representative sub samples (10 g) were taken and used in the study. All samples were stored at 4 °C until analysis.

2.2. Apparatus

A centrifuge Heraeus, Megafuge 1.0 R (Hanau, Germany) and a Brix refractometer 0–90% (ATAGO, Tokyo, Japan) were used.

The HPLC was performed on a Thermo-Finnigan (81 Wyman Street, Waltham, MA 02454, USA) system which constituted from a PC with a program suitable for the analysis monitoring and peak integration, a degasser (SCM100), a pump (P4000), an autosampler (AS3000), a system controller (SN4000) and a diode-array detector (UV 6000LP). The HPLC column was C_{18} polar endcapped Synergi Hydro-RP, Phenomenex (411 Madrid Avenue Torrance, CA 90501-1430, USA) (4.6 × 25 mm, 4 μm, 80 Å, carbon load 19%). The precolumn used was C_{18}, Alltech (Lexington, Kentucky, USA) (4.6 × 7.5 mm, 5 μm, 100 Å).

In addition the GC-MSD was performed on a SHIMADZU (Kyoto, Japan) gas chromatographer system (GC-2010) connected with a PC with a program suitable for the analysis monitoring and peak integration, an autosampler (AOC20s), an autoinjector (AOC20i) and a mass spectrometer (GC-MS QP2010). The GC column was 50% phenyl, 50% methyl-polysiloxane, SGE, (30 × 0.25 mm, 0.25 μm).

2.3. Reagents

Patulin standard solution (100 μg mL^{-1} in chloroform) was purchased from Supelco, Pennsylvania, USA). The ethyl acetate (purity 99.8%), anhydrous sodium sulfate and perchloric acid (70% w/v) were from Riedel de Haen, (Seetze, Germany). The sodium carbonate (solution 1.5%) was from Fluka Chemika (St. Galen, Switzerland). The acetic acid 65% w/v (solution pH 4) was from Merck (Darmstand, Germany). The chloroform was from Mallinckrodt (675 McDonnell Blvd., Hazelwood MO 63042, USA). The Nitrogen and the Helium were of purity ECD. and the *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) was from Sigma–Aldrich (St. Luis, USA). All reagents used were of analytical grade while HPLC solvents (Acetonitrile) were HPLC gradient grade and purchased from Lab-Scan (Dublin, Ireland).

2.4. Samples procedure for the HPLC-DAD method

Transfer 10 g of the sub sample to a plastic cap covered centrifuge tube (50 mL). Add 10 mL ethyl acetate, and shake the tube vigorously for 1 min using Vortex mixer. Centrifuge the mixture at 4500 rpm (3509*g*), at 25 °C, for 3 min. Transfer the upper organic layer to another centrifuge tube. Repeat the extraction with 10 mL and with 5 mL ethyl acetate and combine the organic layers. Add 2 mL 1.5% Na_2CO_3 solution to the combined ethyl acetate layers and shake the tube vigorously using Vortex mixer for 30 s (Gökmen & Acar, 1999). Repeat the previously described procedure twice. Carry out the second and the third centrifugation at 4000 rpm (2772*g*) and 3000 rpm (1559*g*), respectively, at 25 °C for 1 min. Then combine

the organic layers and dry with anhydrous Na_2SO_4 because patulin could be destroyed if aqueous ethyl acetate extract was evaporated to dryness. Place the tube in a heating block at 40 °C and evaporate to 1–2 mL under stream of N_2. Quantitatively transfer the extract to a vial (8 mL), rinsing the tube with ethyl acetate, and complete the evaporation to dryness. Finally dissolve the residue in 1 mL acetic acid solution (pH 4) and analyze with HPLC-DAD.

2.5. Determination of patulin with the HPLC-DAD method

The operating conditions for the determination of PAT by HPLC-DAD method were: the mobile phase, water + acetonitrile + perchloric acid (990 + 10 + 1) was filtered through Millipore (290 Concord Road Billerica, MA01821, USA) HA VLP (0.45 μm) filters before using it, The injection volume was 50 μL. The detection of patulin was performed by scanning from 220 to 360 nm. Maximum absorption of patulin was at $\lambda = 276$ nm. The flow rate was 1 mL min^{-1} and the retention time (RT) was 26 min.

2.6. Samples procedure for the GC-MSD method

The procedure for patulin extraction from juices is described in Section 2.4 and it is a method common for the GC-MSD as well. A volatile derivative of PAT (trimethylsilyl-patulin) is obtained by a substitution nucleophilic bimolecular reaction (S_N2) as following. Evaporate 1–2 mL of the ethyl acetate under N_2, is transfer quantitatively the extract to a vial (8 mL), rinse the tube with ethyl acetate, and complete the evaporation to dryness. Dissolve the residue in 1 mL acetonitrile, take 300 μL of it and mix with 100 μL BSTFA in a 2 mL vial with a cap. Shake the vial vigorously for 1 min. Heat the mixture at 70 °C for 15 min and analyze with GC-MSD.

2.7. Determination of patulin with the GC-MSD method

The operating conditions for the determination of PAT by HPLC-DAD method were: electron impact ionization; ionization energy 70 eV; injection volume 1 μL; helium carrier gas at constant flow of 1.42 mL min^{-1}; injector temperature at 280 °C; initial oven temperature at 100 °C for 2 min, ramp at 10 °C min^{-1} to 200 °C and then 20 °C min^{-1} to 300 °C, hold for 3 min; splitless injection of 1 μL; detector's temperature at 300 °C; solvent delay for 2 min. The monitor for selected ions m/z were 136, 155, 170, 183, 211, 226. The retention time of patulin was 10.6 min and the quantification done by integration of the peak of ion m/z were 136.

3. Results and discussion

3.1. Characterization of the methods HPLC-DAD and GC-MSD

The analytical protocol for the determination of patulin for both methods was in-house characterized regarding the following criteria: linearity, accuracy, repeatability and internal reproducibility, limits of detection (DL) and quantification (QL).

Additionally, the limits of repeatability (r) and reproducibility (R) were calculated as following: $r = 2.8 \times SD_r$ and $R = 2.8 \times SD_R$, respectively. With regard to the limits of detection, DL and quantification QL were calculated according to: $DL = [b_o + 3S(b_o)]/b_1$, $QL = [b_o + 10S(b_o)]/b_1$, where b_o is the response of the blank (intercept of the calibration model), $S(b_o)$ is the standard deviation of the blank and b_1 is the sensitivity (calibration model slope). The accuracy was estimated by analyzing samples (10 g) of apple juice, spiked with different quantities of patulin. Additionally, a Fisher test was applied to confirm the acceptability of the linear regression. If the Fl ratio is greater than the critical value $F(1, p(n-1), 1-a)$ corresponding to a Fisher variable with a risk $a = 0.1\%$ for 1 and $p(n-1)$ degrees of freedom, the regression model can be considered as acceptable. Then the hypothesis must be checked if the lack of fit of the model is negligible and that it is a straight line throughout the chosen field. If the Fl ratio is less or equal to the critical value $F[p-2, p(n-1), 1-a)$ corresponding to a Fisher variable with a risk of 0.1% for $p-2$ and $p(n-1)$ degrees of freedom, the field of linearity chosen can then be approved. For both methods $p = 5$ levels and $n = 4$ repetitions were used.

3.2. Characterization of the HPLC-DAD method

With regard to linearity, a solution of 1 μg mL^{-1} in acetic acid solution (pH 4) was prepared from a stock solution of patulin 100 μg mL^{-1} in chloroform. Then solutions of patulin were prepared at concentrations 50, 100, 150, 250 and 500 ng mL^{-1}. The volume injected onto the column was 50 μL. Linear regression was used to prepare standard curves by using the mean values of peak areas of five injections of the five solutions. The equation of the standard curve was

$$E = 25.8(\pm 0.4) \times 10^3 C - 2.2(\pm 5.58) \times 10^4$$

$$r = 0.99995 \ \mathrm{RSD}\% = 0.52$$

where E is the peak area of patulin injected (50 μL) and C is the ng of the standard patulin injected (50 μL).

With regard to repeatability, this parameter was estimated by analyzing six samples (10 g) of apple juice spiked with 0.5 μg of PAT corresponding to 50 kg^{-1} of juice under repetitive conditions. The coefficient of variation determined was found to be 3.7% (mean = 0.048 μg kg^{-1}). The internal reproducibility was estimated by determining (at time intervals = at six different days of the month) the recoveries of six samples (10 g) of apple juice spiked with patulin (0.020, 0.050, 0.100, 0.150, 0.250, 0.500 μg). The mean recovery during the reproducibility experiments was found to be 97.0% (RSD% = 4.1). Additionally, the limits of repeatability (r) and reproducibility (R) were found to be $r = 4.28$ and $R = 11.2$ ($SD_r = 3.6$ and $SD_R = 4$), respectively.

The accuracy of the method applied in apple juice was studied by analyzing samples spiked at different concentrations as being shown in Table 1. The regression coefficient r of the curve was found to be $r = 0.9999$, [$y = 0.995\ (\pm 0.023)\ x - 0.18\ (\pm 0.61)$], where y is the concentration of patulin ($\mu g\ kg^{-1}$) of apple juice recovered and x is the concentration of patulin ($\mu g\ kg^{-1}$) of apple juice spiked. Applying the Fisher test the found $F(1, 15)$ ratio of 18353.7 was greater than the critical Fisher value of 16.59 at an alpha risk 0.1% for 1 and 15 degrees of freedom. Therefore, the regression model can be considered as acceptable. Finally, the lack of fit of the model was found to be lower as the experimental F ratio (2.59) is less than the critical value $F(3, 15) = 9.335$ with a risk $a = 0.1\%$ for 3 and 15 degrees of freedom. Therefore, the field of linearity chosen can then be approved.

The mean recovery of the method calculated from the above equation was found to be 99.5% (RSD = 0.73%). The DL was determined at 0.23 $\mu g\ kg^{-1}$ of apple juice and the QL at 1.2 $\mu g\ kg^{-1}$ of apple juice.

Gökmen and Acar (1999) reported a HPLC-DAD method for PAT determination in apple juice with 94% recovery and detection limit 5 $\mu g\ kg^{-1}$ as the limit level resulted from a signal to noise at a ratio of 3:1. Additionally Li, Wu, Hu and Wang (2007) reported a method for the determination of PAT using an HPLC-UV detection with recoveries 94–114% and DL = 5 $\mu g\ kg^{-1}$. According to Bartolome et al. (1994) the DL was determined at 8.96 $\mu g\ kg^{-1}$ and the recoveries ranged from 82.3% to 92.0%. In the present study the DL (0.23 $\mu g\ kg^{-1)}$ and the recovery (99.5%) are more satisfactory.

3.3. Characterization of the GC-MSD method

With regard to linearity, a solution of 1 $\mu g\ mL^{-1}$ in acetonitrile was prepared from a stock solution of patulin 100 $\mu g\ mL^{-1}$ in chloroform. Then solutions of patulin were prepared at concentrations 50, 100, 150, 250 and 500 ng mL^{-1}. The volume injected onto the column was 1 μL. Linear regression was used to prepare standard curves by using the mean values of peak areas of five injections of the five solutions. The equation of the standard curve was

$$E = 35.62(\pm 1.59) \times C - 6.83(\pm 4.2)10^2$$
$$r = 0.9997\ \mathrm{RSD}\% = 1.4$$

where E is the peak area of patulin injected (1 μL) and C is the concentration (ng mL^{-1}) of standard patulin solution injected (1 μL).

With regard to repeatability, this parameter was estimated by analyzing six samples (10 g) of fresh apple juice spiked with 0.5 μg of PAT corresponding to 50 μg PAT kg^{-1} of juice, under repetitive conditions. The coefficient of variation determined was found to be 6.67% (mean = 0.02 kg^{-1}). The internal reproducibility was estimated by determining the recovery (at time intervals = at six different days of the month) of six samples (10 g) of apple juice spiked with patulin (0.05, 0.100, 0.150, 0.250, 0.500 μg). The mean recovery during the reproducibility experiments was 55.3% (RSD% = 23.2). Additionally, the limits of repeatability (r) and reproducibility (R) were found to be $r = 8$ and $R = 36.12$ ($SD_r = 2.86$ and $SD_R = 12.9$), respectively.

The accuracy was estimated by analyzing samples (10 g) of apple juice, spiked with different quantities of patulin. As being shown in Table 2, a satisfactory linear relationship was established between quantities spiked and quantities recovered.

The regression coefficient r of the curve was found to be $r = 0.985$, [$y = 0.41\ (\pm 0.13)\ x - 1.02\ (\pm 3.48)$], where y is the concentration of PAT ($\mu g\ kg^{-1}$) of apple juice recovered and x is the concentration of PAT ($\mu g\ kg^{-1}$) of apple juice spiked. Applying the Fisher test the found $F(1, 15)$ ratio of 99.04 was greater than the critical Fisher value of 16.59 at an alpha risk 0.1% for 1 and 15 degrees of freedom. Therefore, the regression model can be considered acceptable. But, the lack of fit of the model was found to be higher as the experimental F ratio (40.77) is more than the critical value $F(3, 15) = 9.335$ with a risk $a = 0.1\%$ for 3 and 15 degrees of freedom. Therefore, the field of linearity chosen cannot be approved at an alpha risk 0.1%.

The mean recovery of the method calculated from the above equation was found to be 41% (RSD = 10.05%). The PAT DL was determined at 5.8 $\mu g\ kg^{-1}$ of apple juice and the QL at 13.8 $\mu g\ kg^{-1}$ of apple juice. Rupp and Turnipseed (2000) reported a GC-MSD method for the

Table 1
Precision and accuracy of the method HPLC-DAD for patulin in apple juice

Samples	Concentration of patulin ($\mu g\ kg^{-1}$) spiked in apple juice				
	5	10	15	25	50
	Patulin concentration ($\mu g\ kg^{-1}$) recovered				
1	4.9	9.7	14.7	24.2	49.2
2	5	9.9	14.8	24.4	50.2
3	4.9	9.9	14.5	24.7	50.2
4	4.9	10.1	14.6	23.9	49.1
Mean	4.9	9.9	14.7	24.3	49.7
(S.D.)	0.05	0.16	0.13	0.3	0.6
(% R.S.D.)	0.05	1.65	0.9	1.4	1.2

$y = 0.995\ (+0.023)\ x - 0.18\ (+0.61)$.
$r = 0.9999$ RSD% = 0.73.

Table 2
Precision and accuracy of the method GC-MSD for patulin in apple juice

Samples	Concentration of patulin ($\mu g\ kg^{-1}$) spiked in apple juice				
	5	10	15	25	50
	Patulin concentration ($\mu g\ kg^{-1}$) recovered				
1	3.1	6.5	6.9	9.6	23.9
2	2.8	6.6	6.6	9.9	21.7
3	3	6.7	6.7	9.6	22.6
4	3	7.2	6.6	9.1	21.6
Mean	3	6.8	6.7	9.6	22.4
(S.D.)	0.12	0.3	0.1	0.4	0.9
(% R.S.D.)	4	4.9	1.7	3.8	4

$y = 0.41\ (+0.13)\ x - 1.02\ (+3.48)$.
$r = 0.985$ RSD% = 10.05.

determination of patulin in apple juice. The presence of patulin was confirmed at levels of about 30–400 μg L^{-1} but no information was available concerning the recovery and the accuracy of the method. The DL of the present study is more satisfactory.

3.4. Comparison of the methods

To our knowledge there is no information concerning the comparison of the HPLC-DAD method with the GC-MSD method. These two methods were compared in order to evaluate the existing systematic errors in the method GC-MSD in comparison with the method HPLC-DAD which is taken into account as a reference. Student's *t*-test was applied for examining the significant difference between the intercept value and zero and between the slope value and the unity. By plotting the quantities of the patulin obtained by determining fruit juice samples spiked with 5, 10, 15, 25, 50 μg PAT kg^{-1}, a satisfactory linear correlation was established between the two methods.

$$y = 0.42(\pm 0.12)x + 1.08(\pm 3.2)$$

$$r = 0.987 \ \mathrm{RSD}\% = 9.39$$

where: $y = \mu g\ kg^{-1}$ of patulin analyzed using the method GC-MSD$x = \mu g\ kg^{-1}$ of patulin analyzed using the method HPLC-DAD.

The *t*-experimental value for the intercept-zero comparison is

$$\frac{1.08}{3.24} = 0.33$$

This is less than *t*-theoretical = 3.182 (degrees of freedom = $N-2 = 3$) for 95% confidence level, showing that there is no constant systematic error.

Conversely, the *t*-experimental for the slope-unity comparison is

$$\frac{1 - 0.41}{0.12} = 4.8 > t\text{-theoretical} = 3.2$$

This shows that between the two methods a systematic error of 58% against the GC-MSD method exists.

Both methods are simple and efficient. However in Table 3 is shown that between the two methods important differences exist. It is obvious that the differences are due to the HPLC-DAD and GC-MSD physicochemical properties and their technical specifications. It has already mentioned that the extraction of patulin from the juices is a common procedure for both methods. The accuracy (recovery 99.5%) of the HPLC method is more satisfactory than the accuracy (recovery 41.3%) of the GC-MSD method. The DL (0.23 μg kg^{-1}) and QL(1.20 μg kg^{-1}) of the HPLC-DAD method are more satisfactory in comparison with the DL (5.8 μg kg^{-1}) and QL (13.8 μg kg^{-1}) of the GC-MSD method. On the other hand, the limits of repeatability (*r*) (4.28 and 4) are very satisfactory for both methods. On the contrary, the limit of reproducibility (*R*) (11.2) by the HPLC is more satisfactory in comparison with the limit *R* (36.1) by the GC method. In addition, the cost of the GC-MSD method is higher than the HPLC-DAD method. However the Rt (10.6 min) of the CG-MSD method is more rapid than the Rt (26 min) of the HPLC-DAD and the resolution of the patulin peak with the GC-MSD method is more satisfactory in comparison with the resolution of the patulin peak with the HPLC-DAD method.

Table 3
Characterization and comparison between the HPLC-DAD and the GC-MSD methods for the determination of patulin in fruit juice

	Analytical methods	
	HPLC-DAD	GC-MSD sim
% Recovery	99.5	41.3
% R.S.D.	0.73	10.05
D.L. (μg kg^{-1})[a]	0.23	5.8
Q.L. (μg kg^{-1})[b]	1.2	13.8
r[c]	4.28	4.0
R[d]	11.2	36.1
Rt[e]	26 min	10.6 min

[a] Detection limit.
[b] Quantification limit.
[c] Limit of repeatability.
[d] Limit of reproducibility.
[e] Retention time.

3.5. Occurrence of patulin in fruit juices and the exposure assessment

In the present study patulin was detected in 100% of the samples examined ($n = 90$). In Table 4 the occurrence of PAT in forty imported concentrated fruit juices is shown. The mean value of PAT in all concentrated fruit juices was found to be 10.54 μg PAT kg^{-1} juice (RSD% = 69).

The mean and median values of PAT occurrence in 23 concentrated apple juices from Turkey were found to be 11.60 and 9.50 μg kg^{-1} juices, respectively. In addition the mean and median values of PAT occurrence in 14 concentrated apple juices from China were found to be 8.60 and 8.50 μg kg^{-1} juices, respectively. Moreover, two concentrated apricot juices from China were contaminated with 12.2 and 15.3 μg PAT kg^{-1} juice and one concentrated pineapple juice was contaminated with 7.7 μg PAT kg^{-1} juice. The most contaminated samples were four originated from Turkey (36.8, 26.8, 24.6, 19.10, 18.10 μg PAT kg^{-1} juice) and one from China (15.3 μg PAT kg^{-1} juice).

Table 4
Occurrence of patulin in imported concentrated fruit juices[a]

Samples	Origin	No.	Range of contamination PAT μg kg^{-1}	Mean PAT μg kg^{-1}	Median PAT μg kg^{-1}
Apples	Turkey	23	2.6–36.8	11.60	9.50
Apples	China	14	3.8–16.2	8.50	8.30
Apricot	China	2	12.2–15.3	13.70	13.70
Pineapple[b]		1	7.7	–	

[a] All concentrated juices were appropriately diluted before analysis.
[b] Unknown origin.

In Table 5 the occurrence of PAT in 50 samples of commercial fruit juices and other fruit's foodstuffs collected from the Greek markets is shown. The mean value of PAT in 46 commercial fruit juices is 5.57 (RSD% = 12). The mean and median values of PAT occurrence in 29 commercial apple juices were found to be 5.50 and 5.0 μg PAT kg^{-1} juice, respectively. The mean and median values in 12 commercial mixed juices were found to be 5.50 and 5.0 μg PAT kg^{-1} juice, respectively. The mean and median values in 12 commercial mixed juices were found to be 5.60 and 4.30 μg PAT kg^{-1} juice, respectively. Three orange juices were found to be contaminated with PAT ranging from 3.1 μg PAT kg^{-1} juices to 10.8 μg PAT kg^{-1} juices. Moreover, the mean value of PAT occurrence in two rosehip juice was 3.5 μg PAT kg^{-1} juices and in one apple soft drink was 3.2 μg PAT kg^{-1} drink. In addition, three samples of baby foods with apple were analyzed and the mean and median values of PAT occurrence were found to be 5.10 and 4.40 μg PAT kg^{-1} of baby food, respectively.

Among the commercial fruit juices the most contaminated were found to be, three apple (11.8, 9.7 and 10.2 μg kg^{-1} juice) one orange (10.9 μg PAT kg^{-1} juice) and two mixed fruits juices (11.2 and 9.4 μg kg^{-1} juice). All results are reported as uncorrected for recovery.

The percentage (100%) of PAT occurrence in the samples examined could be explained by the low DL (0.23 μg PAT kg^{-1} juice) of the analytical method (HPLC-DAD) used in the present study. On the other hand, the concentrations of PAT determined in all samples were below the limit 50 μg PAT kg^{-1} which is the maximum permitted level of PAT in fruit juices, nectars, apple juices and apple juice's ingredients in other beverages marked in Europe (European Commission, 2003). The permitted level in solid apple products is 25 μg PAT kg^{-1} and the threshold is lower for apple juices intended for children (10 μg PAT kg^{-1} juice).

In the present study, baby foods presented patulin contents below the maximum permitted limit 10 μg PAT kg^{-1} baby foods. The most contaminated sample was found to contain 6.9 μg PAT kg^{-1} baby foods (Table 5).

Previous studies found that PAT occurred in high incidence ranging from 21% in Brazil (Manchisky & Midio, 1996) to 100% in Turkey (Gökmen & Acar, 2000). In the present study 23 concentrated apple juices, positive for PAT, were imported from Turkey. Recently, Piemontese et al. (2005) reported that PAT was detected in 26% of the conventional fruit juices from Italy, showing that only one sample exceeded the maximum permitted level (50 μg PAT kg^{-1} juices). The lower percentage of positive samples reported by Piemontese et al. (2005) could be explained by the higher limit of quantification 0.5 μg kg^{-1} for juices, in comparison with the DL (0.23 μg kg^{-1} juice) used in the present study.

As far as the toxicity of PAT is considered as genotoxic but no adequate evidence for carcinogenicity in experimental animals exists, the Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives (JECFA) have established a provisional maximum tolerable daily intake (PMTDI) of 0.4 μg PAT kg^{-1} bw for PAT (WHO, 1995).

The most frequently PAT-contaminated foods are apples and their derivatives. Fruit juices are often included in a diet important differences as far as the intake is considered .This has to do with the age and the preferences of the consumers. In Table 6 the daily intake of patulin according to the age of Greek consumers is shown. Plunkett, Turnbull and Rodricks (1992) indicate a very high consumption of apples such as (or as their derivatives) in the first years of life (6.4 g kg^{-1} bw per day) with an intake declining with age. This information is in agreement with our estimation of fruit juice consumption (Table 6). The differences observed in food's intake (per unit of body weight) from children and adults mean that for a given concentration of a contaminant, a child will receive a different exposure than an adult will, consuming the same amount of an apple. Consequently in Table 6 is shown that a consumption by a child (20 kg) of 0.25 kg from the most contaminated (36.8 μg PAT kg^{-1}) fruit juice found in the present work, leads to a PAT daily intake (0.46 μg PAT kg^{-1} bw) that exceeds the PMTDI (0.40 μg PAT kg^{-1} bw). On the contrary, a consumption

Table 5
Occurrence of patulin in commercial fruit juices collected form the Greek market

Samples of fruit juices	No.	Range of contamination PAT μg kg^{-1}	Mean PAT μg kg^{-1}	Median PAT μg kg^{-1}
Apples	29	0.9–11.8	5.50	5.0
Orange	3	3.1–10.8	6.80	6.4
Mixed fruits (apple included)	12	2.8–11.2	5.60	4.30
Rosehip	2	3.5–3.6	3.55	3.55
Soft drink[a]	1	3.2	–	–
Baby foods[b]	3	4–6.9	5.10	4.40

[a] Apple.
[b] Apple pulp.

Table 6
Daily intake of patulin[a] (μg PAT kg^{-1} bw)

Age Body weight (kg)	Children 20	Adolescent 55	Adults 70
Consumption fruit juices (kg)[b]	0.1–0.25	0.1–0.5	0.1–0.25
Median 8.9 μg PAT kg^{-1}[c]	0.04–0.1	0.016–0.08	0.01–0.03
Median 4.7 μg PAT kg^{-1}[d]	0.02–0.05	0.008–0.042	0.01–0.02
Most contaminated juice 36.8 μg PAT kg^{-1}[e]	0.18–0.46	0.067–0.33	0.05–0.13
Baby foods Median 4.40 μg PAT kg^{-1}	0.02–0.05	–	–

[a] MTDI 0.4 μg PAT kg^{-1} bw.
[b] Estimated daily consumption by consumers of fruit juices.
[c] Occurrence of PAT in imported juices.
[d] Occurrence of PAT in commercial juices collected from the Greek Market during 2004–2006.
[e] Apple juice imported from Turkey.

of 0.25 kg from the same fruit juice by an adult (70 kg) displayed a daily intake of 3-fold lower from the PMTDI.

Piemontese et al. (2005) reported that the daily intake of PAT in different age's groups in Italy ranged from 0.22 to 3.41 ng kg^{-1} bw with a mean consumption of 21 g per day. A much greater mean to patulin exposure was estimated by the US Food and Drug Administration, Center for Food Safety and Applied Nutrition, as the mean daily intake of PAT was 0.14 μg kg^{-1} bw for all ages and 0.8 μg kg^{-1} bw for 1–2 years old, based on mean apple juice's intakes of 200–216 g daily (FDA/CFSAN, 2001).

The minimum amount of fruit apple juice sold in Greece is ordinarily a small pack of 0.2 L and the maximum is a pack of 1 L. In Table 6 is shown that the estimated daily intakes of fruit juices are established according to the age of the consumers. Fruit juices are very popular to certain Greeks aging from 12 to 18 years old. Therefore, in Table 6 is shown that the daily intake of patulin is very low for all ages consuming either imported or commercial fruit juices with the exception of the case mentioned above. Even the daily intake of patulin from children consuming baby foods is from 0.02 μg PAT kg^{-1} bw to 0.05 μg PAT kg^{-1} bw day. With the exception of the most contaminated with PAT sample, the daily intake for all ages and kinds of fruit juices is ranging from 0.008 μg PAT kg^{-1} bw to 0.1 μg PAT kg^{-1} bw estimating the daily consumption from 0.1 to 0.5 kg of a fruit juice or a fruit's product.

4. Conclusion

In conclusion the good precision, repeatability, reproducibility, the low detection limit and the lower cost make the HPLC-DAD method more convenient for the routine analysis and for the research purposes. However the good repeatability, the very good resolution of patulin with the GC-MSD method and the mass spectrum make it convenient for working out identification's problems associated with the analysis of patulin in fruit juices.

In addition, the risk assessment shows that with a standard diet, the consumer of juices in Greece would receive an amount of patulin below the tolerable level, as long as all commercial products respect the established safe limits. On the other hand, excessive intake could occur in the case of children consuming every day high quantities of apple juices or mixed, with apple, fruit juices.

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