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Evaluation of Patulin in Commercial Baby Foods by solid Phase Extraction and Liquid Chromatography PDA Detection

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Abstract

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A simple, reliable, and rapid RP-LC method has been developed for the determination of patulin in commercial baby foods from Turkish markets. Solid phase extraction and liquid chromatography with photodiode array detection technique were used for the analysis of patulin in samples. Linearity was obtained in different concentration ranges between 0.001 and 0.050 µg/l. The recovery rates for patulin ranged from 92.85% to 100.45% with a coefficient of variation less than 3.0%. LOD and LOQ of the method were found to be 9.66×10^{-6} and 2.93×10^{-5} µg/ml, respectively. The proposed method has been extensively validated in accordance with ICH guidelines. It can be used by routine analysis laboratories within a short time of analysis. All of the detected samples containing patulin were below the level suggested by the World Health Organization.

Keywords: homogenised puree; HPLC; patulin; Turkish markers; SPE

Patulin (PAT, 4-hydroxy-4H-furo[3,2-c]pyran-2(6H)-one) is a mycotoxin produced by a wide range of fungal species of the genera *Penicillium*, *Aspergillus*, *Byssochlamys*, *Eupenicillium*, and *Paecilomyces*, of which *Penicillium expansum*, a common contaminant of damaged fruits, is the most important (Fuchs *et al*. 2008). It has been identified in different types of fruits (apples, pears, peaches, cherries, black currants, oranges, apricots, pineapple, grapes, bananas, strawberries, plums) (Pittet 2001), but it has found to a larger extent in apples, pears, peaches, and in their processed products like juices and purées (WHO Report 1995).

PAT has been reported to be mutagenic and to cause neurotoxic, immunotoxic, genotoxic, and gastrointestinal effects in rodents (Hopkins 1993). Thus, there is some concern that similar effects may occur in humans, through the long-term consumption of foods and beverages contaminated by this mycotoxin. The International Agency for Research on Cancer (IARC) has classified PAT as category 3, not classifiable regarding its carcinogenicity to humans (IARC 1993). Due to its toxicity, the Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives (JECFA) established a provisional maximum tolerable daily intake (PMTDI) for PAT of 0.4 mg/kg body weight/day (WHO Report 1995). Because of human health concerns the European Commission established a maximum concentration of 50 mg/kg of PAT in fruit juices and fruit nectars, reconstituted fruit juices, spirit drinks, cider and other fermented drinks derived from apples or containing apple juice, 25 mg/kg for solid apple products, while the maximum level allowed for apple products intended for infants and young children is 10 mg/kg (European Commission Regulation 2006).

5-Hydroxymethylfurfural (HMF) and PAT exhibit similar chromatographic properties owing to their chemical structures (Table 1), and therefore HMF appears to be the most commonly encountered interference during the liquid chromatographic analysis of patulin (Gokmen & Acar 1996). Both HMF and PAT are important quality criteria in many kinds of foods. Also, the presence of HMF is considered as an indication of deterioration. It is formed as a result of dehydration of ketopentoses, particularly in acidic or high-temperature environments (Alfonso *et al*. 1980; Lee *et al*. 1986).

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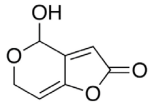
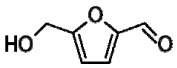
Table 1. Chemical structure of the studied compounds

Compound	Chemical structure		
Patulin (PAT) 4-hydroxy-4H-furo[3,2-c]pyran-2(6H)-one CAS No. 149-29-1		$C_7H_6NO_4$	MW: 154.12 g/mol
5-hydroxymethylfurfural (HMF) 5-(hydroxymethyl)-2-furaldehyde CAS No. 67-47-0		$C_6H_6O_3$	MW: 126.11 g/mol

Several researchers have reported the confirmation of patulin (in apple juice) by GC/MS following the formation of a derivative of patulin (Koach *et al*. 2000). However, quantification is still based on LC rather than on GC/MS. The most widely used method to analyse patulin has been reversed-phase HPLC, since patulin is a polar compound (Shepard & Leggott 2000; Llovera *et al*. 2005). The majority of the reported methods exhibited poor selectivity due to co-elution of patulin and 5-HMF. Although only one method has been found for a possibility of simultaneous determination of these compounds in apple (Gokmen & Acer 1999), there is no report on determination of these constituents in homogenised baby foods.

The homogenised baby foods (both meat and vegetables, and fruits) are considered to be food ready for use, sterilised and hermetically packaged, prepared from properly controlled nutrients, subjected to a homogenisation process to improve the ingestion and digestion. In view of their wide use, both because they supply the nutritional needs for children and because they combine easiness and rapidity in preparing meals, several samples of fruit-homogenised baby food were collected from Turkish markets to analyse and assess the patulin presence and their compliance with the limits laid down by the law. This work describes, for the first time, an improved, new, rapid, selective, accurate, precise, sensitive, fully validated SPE-LC-DAD method to quantify the patulin in fruit-based baby foods (homogenised purées and infant drink) commercially available in the Turkish markets.

MATERIAL AND METHODS

Material. A total of 6 fruit-based baby food (3 mixtures of homogenised apple, carrot, grape and banana purées and 3 pure and mixed apple juices) products were purchased in different Turkish supermarkets in 2013. Each sample was extracted in duplicate with triplicate injections into the column.

Apparatus. The LC analysis was carried out on a Shimadzu HPLC system with a pump (LC-20 AD), DAD detector system (SPD-M 20A) and column oven (CTO 20 AC). This equipment has a degasser system (DGU 20 A). The system operates at 276 nm for PAT and 282 nm for HMF. The analytical columns obtained from Phenomenex-Gemini C18 (110 Å, 250 mm × 4.6 mm × 3 µm), Synergy Hydro RP (80 Å, 250 mm × 4.6 mm × 4 µm) and Luna C18(2) (100 Å, 250 mm × 4.6 mm × 5 µm) were used as stationary phases at 40°C. Mettler Toledo MA 235 pH/ion analyser with Hanna HI 1332 Ag/AgCl combined glass electrode was used for pH measurements.

To determine the exact mass concentration, Biochrom Libra S70 (Cambridge, UK) UV-visible spectrophotometer was used and recorded the absorption curve between 250 and 350 nm in a 1 cm quartz cell with ethanol as reference. The material for solid phase extraction (SPE) was a Supelco SPE system and vacuum pump with Waters-OASIS HLB 1 ml cartridges (Waters Corp., Milford, USA).

Chemicals and reagents. All chemicals and solvents were of analytical reagent grade and used without further purification. Ethyl acetate (extra pure), acetonitrile (ACN, HPLC grade), acetic acid (extra pure), sodium carbonate (reagent grade) and anhydrous sodium sulphate (reagent grade), sodium hydroxide, potassium bromide, *n*-hexane, ethanol, perchloric acid, and sodium bicarbonate were obtained from Sigma-Aldrich (St. Louis, USA). Potassium hydrogen phthalate (dried at 110°C before use; Fluka, St. Gallen, Switzerland) was used. All stock solutions were prepared in distilled water. Water, with conductivity lower than 0.05 m/Scm, was obtained with Zeneer Power I (Human & Technologies Corp., Seoul, South Korea).

Stock solution of HMF was prepared by dissolving 10 mg of pure HMF (Sigma-Aldrich, St. Louis, USA) in 50 ml of distilled water. Stock solution of patulin

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was also prepared by dissolving 5 mg of pure crystalline patulin (Sigma-Aldrich, St. Louis, USA) in ethyl acetate. Transfer the solution to a 25 ml volumetric flask and dilute the volume with ethyl acetate to produce a solution containing approximately 200 µg/ml of patulin. This solution was stored in a freezer at approximately –18°C. Confirmation of the mass concentration of the solution is done if it is older than six weeks. A solution stored in this way is stable for several months. Ensure the solution is allowed to reach room temperature before use to avoid the incorporation of water by condensation.

Patulin standard solution. The respective concentrations of patulin were calculated according to the method described by European Standard EN 15890 (European Standard Report 2010). 1000 µl of the stock solution was evaporated to dryness under nitrogen and then immediately dissolved in 20 ml of ethanol to obtain a mass concentration of approximately 10 µg/ml of patulin. The absorption curve was recorded between 250 nm and 350 nm in a 1 cm quartz cell with ethanol as reference in order to determine the exact mass concentration. Calculation of the mass concentration of patulin, ρ_{pat}, in micrograms per millilitre, was found by using Equation 1;

$$\rho_{pat} = \frac{A_{max} \times M \times 100}{\varepsilon \times b} \quad (1)$$

where: A_{max} – absorption determined at the maximum of the absorption curve (approximately 276 nm); M – molar mass; ε – molar absorption coefficient of patulin in ethanol (1460 m^2/mol); b – optical path length of the quartz cell

This solution was stored in a freezer at approximately –18°C. A solution stored in this way is stable for several months.

Chromatographic procedure. Throughout this study, the mobile phases assayed were ACN-water mixtures at 5, 10, and 15 % (v/v). The pH of the mobile phase was adjusted to 2.5, 3.0, and 4.0 by the addition of 1 mol/l sodium hydroxide with the presence of different buffer components. It was filtered through a 0.45 mm regenerated cellulose acetate membrane (Millipore, Bedford, USA) and degassed by an ultrasonic bath. The flow rate was maintained at 1.0 ml/min and the injected volume was 20 µl.

The column was pre-conditioned during 1 h at least at a low flow rate (0.5 ml/min) with mobile phase at the corresponding pH before the first injection. For each compound, the retention time values – t_R were determined from three separate injections for each mobile phase composition and pH considered.

The chromatographic retention of ionisable compounds is strongly dependent on the pH of the mobile phase. Thus, an accurate measurement and control of mobile phase pH is required, in many instances, for efficient separations of ionisable compounds by RP-LC (Rizzi *et al*. 1998).

Sample procedure and SPE. The samples of fruit-homogenised baby food were analysed using the extractive method described by Bonerba *et al*. (2010) according to the method of Brause *et al*. (2010) with some modifications. 10 g of the product from each sample collected were placed in centrifuge tubes and diluted with 10 ml of water. The samples were extracted with 3 different volumes of ethyl acetate (50, 25, and 15 ml), vigorously shaken on a Vortex for 10 minutes. The organic supernatant was separated, and 9 ml of 1.5% (w/w) sodium carbonate solution were added. The combined ethyl acetate layers were shaken vigorously. After separation of the 2 phases, the bottom phase (sodium carbonate solution) was extracted with 10 ml ethyl acetate. The organic phase was combined with the ethyl acetate layer of the previous extraction. Dehydration was performed with 4 g of anhydrous sodium sulphate; organic extract was evaporated to dryness using a rotary evaporator.

The dry residue was dissolved in 2.0 ml of chloroform and was loaded onto Waters Oasis hydrophilic lipophilic balanced (HLB) 1 ml cartridge (30 mg sorbent per cartridge, 30 µm particle size) previously conditioned with 1 ml of methanol and 1 ml of water. 1 ml sample was transferred onto the column and allowed to percolate at 2–3 ml/min, under gentle suction. The SPE column was washed with 1 ml of 1% sodium bicarbonate, then 1 ml of 0.1% acetic acid under gentle suction. The column packing dried under vacuum conditions for 3–4 minutes. Finally, 1 ml of ethylacetate/*n*-hexane (3 : 2 v/v) was passed twice through the column and the eluate was collected. Samples were evaporated to dryness with nitrogen gas, dissolved in 0.2 ml mobile phase, and filtered on cellulose filters 0.45 µm. Aliquots of 20 µl of the filtrate were then injected into the HPLC system.

RESULTS AND DISCUSSION

Separation efficiency. Various mobile phase systems were used to provide an appropriate LC separation (ACN-water mixtures at 5, 10, or 15% v/v). Three

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Table 2. Comparison of three columns

	HMF			Patulin		
	1st column[a]	2nd column[b]	3rd column[c]	1st column[a]	2nd column[b]	3rd column[c]
Retention time (min)	6.692	6.771	6.652	10.304	8.644	8.942
Theoretical plates (*N*)	23 672	18 374	1 688	10 889	5479	204
Tailing factor (T)	1.241	1.293	1.543	0.777	–	0.580

[a]Gemini C18, 110 Å, 250 mm × 4.6 mm × 3 µm; [b]Synergy Hydro RP, 80 Å, 250 mm × 4.6 mm × 4 µm; [c]Luna C18(2) 100 Å, 250 m × 4.6 mm × 5 µm

different types of columns such as Luna C18 100 Å (250 mm × 4.6 mm × 5 µm), Synergy Hydro RP 80 Å (250 mm × 4.6 mm × 4 µm) and Gemini C18 110 Å (250 mm × 4.6 mm × 3 µm) were tested in order to find the best resolution and peak shape of the studied compounds. As can be seen from Table 2, the Gemini C-18 column has been selected and used successfully as a stationary phase for the simultaneous determination and separation of PAT and HMF due to the better tailing factor (T) and theoretical plates (N). This column is a C18 bonded phase endcapped with TMS. This stationary phase has extended pH stability (pH 1–12) and is more stable and more efficient than classical packing. The silica surface area is 375 m^2/g with carbon loading of 14%.

In order to find the optimum chromatographic conditions for RP-LC determination of HMF and PAT, the effect of organic modifier, the influence of pH on the mobile phase and column temperature were examined. Mobile phases containing 15% (v/v) ACN as the organic modifier were shown to improve peak performance, retention time, and peak symmetry for the studied compounds. Different kinds of buffer components were tested and acetic acid was found to be more suitable than perchloric acid. The pH of the mobile phase was always adjusted to be 4.0 with 15 mM acetic acid, which is optimum pH with the best peak asymmetry and retention values. The column temperature was set between 25 and 40°C. A higher temperature was shown to minimise the tailing of the compounds. 40°C was selected due to shorter analysis time and improved peak shapes. Finally, the ACN/water 15 : 85 (v/v) mobile phase with 15 mM CH_3COOH (pH 4.0) at a flow rate of 1.0 ml/min was chosen as the most suitable carrier for LC analysis.

Table 3. System suitability parameters and statistical evaluation of calibration data of the studied compounds

	Patulin	HMF
Retention time (min)	5.720	4.816
Tailing factor (T)	1.299	1.312
Capacity factor (*k*)	0.594	0.324
Resolution (R)	4.194	–
Theoretical plates (*N*)	23484	20864
Selectivity factor	1.737	-
RSD% (for retention time)	0.032	0.023
Linearity range (µg/ml) (*n* = 6)	0.001–0.050	
Slope	74.376	
Intercept	0.0656	
Standard error of slope	1.4317	
Standard error of intercept	0.0322	
Correlation coefficient	0.9992	
Detection limit (LOD) (µg/ml)	9.662×10^{-6}	
Quantitation limit (LOQ) (µg/ml)	2.928×10^{-5}	

The method was validated according to the International Conference on Harmonization (ICH) guidelines for analytical method validation (ICH Q2(R1) 2005). The calibration curve and equation for patulin were calculated by plotting the peak area ratios of PAT to HMF (0.025 µg/ml) versus the concentration of the compounds (Table 3). The LOD and LOQ were calculated from the subsequent equations, and using the standard deviation (s) of the response and the slope (m) of the corresponding calibration curve (Riley & Rosanske 1996; Swartz & Krull 1997), LOD = 3.3 s/m, and LOQ = 10 s/m.

Precision and reproducibility of the method were performed by doing the replicate analysis of standard solutions in the mobile phase. Repeatability and reproducibility were characterised by mean recovery and RSD and the summary of the results is given in

Table 4. Summary of repeatability (intra-day) and reproducibility (inter-day) of precision data for patulin

Patulin (µg/ml)	Intra-day	Inter-day
	mean recovery* ± RSD (%)	
0.005	98.40 ± 1.22	92.85 ± 4.22
0.020	100.45 ± 1.13	95.81 ± 8.58

*each value is obtained from five experiments (*n* = 5)

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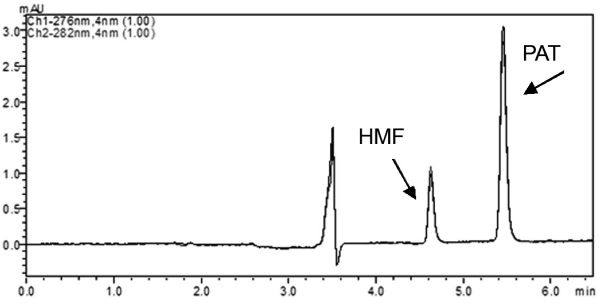

Figure 1. Chromatogram of a standard mixture of the studied compounds containing 0.05 µg/ml HMF and 0.025 µg/ml patulin

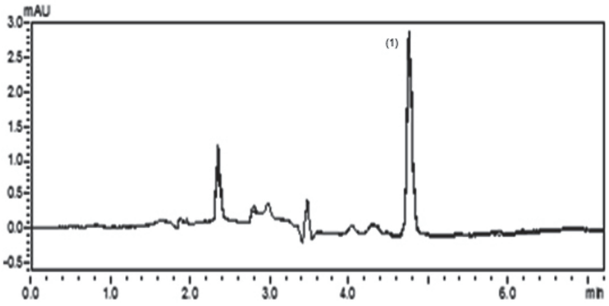

Figure 2. Homogenised fruit juice chromatogram (34.03% grape, 17% apple, 10% carrot, 4% banana)
(1) HMF

Table 4. As can be deduced from Table 3, there was no significant difference for the assay, as tested by within-day and between days.

Baby food samples were spiked with two different concentrations of patulin. The determined results and recoveries of known amounts of patulin added to baby food samples are given in Table 5. The proposed method gives reproducible results, is easy to perform and is sensitive enough to determine patulin in baby foods.

The proposed method has several advantages compared to the standardised method for patulin in foods for infants and young children (European Standard Report 2010). This method provides a simple procedure for the simultaneous analysis of PAT and HMF in food samples by DAD detection at 276 and 282 nm, respectively. Figure 1 shows a chromatogram of the separation of mixtures of PAT and HMF using the conditions described above at 276 and 282 nm. As shown in Figure 1, the studied compounds were forming well shaped symmetrical single peaks, perfectly separated from the solvent front and each other in a short time of analysis (< 6 min). The separation time in European Standard EN 15890:2010 is longer than 12 min and the use of perchloric acid as a buffer component poses serious problems such as twin and wide peaks. Also, the obtained recoveries are better than literature values and standards. The use of the proposed LC method was verified by means of replicate estimations of the samples of fruit-homogenised baby foods.

Applicability. The applicability of this method was tested analysing 6 fruit-based baby foods (3 mixtures of homogenised apple, carrot, grape, and banana purées and 3 pure and mixed apple juices). HMF could easily be detected in most of the samples using the proposed LC method. The presence of relatively higher concentrations of HMF in the samples did not influence the measurement of patulin. Although as low as 1 µg/l of patulin in samples could easily be detected without any disadvantage, no significantly higher concentration of patulin was detected in the studied samples. The patulin concentration found in all samples was included ≤ 3 µg/kg. Therefore all of the analysed samples may be considered as in accordance with product and safe standards required. Figure 2 also illustrates the commercially available homogenised fruit juice chromatogram.

CONCLUSIONS

It can be concluded from the results that the proposed method is faster, easier, reliable and more inexpensive, so it can be used by government agencies,

Table 5. Results obtained for patulin from baby foods using the HPLC method

Patulin concentration (µg/ml)	Found	Bias (%)	Patulin concentration (µg/ml)	Found	Bias (%)
	0.02019	0.95		0.00491	−1.80
	0.01984	−0.8		0.00451	−9.80
0.020	0.02067	3.35	0.005	0.00499	−0.20
	0.01985	−0.75		0.00487	−2.60
	0.02035	1.75		0.00456	−8.80
Mean recovery	0.02018			0.00477	
RSD (%)	1.74075			4.56714	

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routine analysis laboratories and manufacturers in monitoring the quality of commercial fruit-based baby foods. The recovery rates for patulin ranged from 92.85% to 100.45% with a coefficient of variation less than 3.0%. LOD and LOQ of the method were found to be 9.66×10^{-6} and 2.93×10^{-5} μg/ml, respectively. High percentage recovery data shows that the proposed LC method is free from the interferences. All of the detected samples containing patulin were below the level suggested by the World Health Organization.

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