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MONITORIZATION OF PATULIN AND HYDROXYMETHYLFURFURAL IN FRUIT JUICES AND COMMERCIAL FRUITY BABY FOODS BY AN HPLC-DAD METHOD

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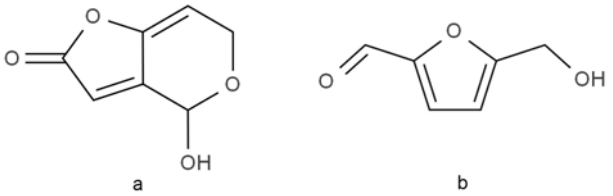
Patulin as a mycotoxin and hydroxymethylfurfural as a chemicals decomposition product are the toxic compounds that are suspected to be cytotoxic and carcinogenic. The aim of this study was to develop a fast, convenient and reliable high-performance liquid chromatography (HPLC) method for the simultaneous analysis of patulin and hydroxymethylfurfural in 86 commercially available fruity baby foods (n=18) and fruit juices (n=68). Fruit juice or baby food sample (5 mL) was extracted with 10 mL ethyl acetate twice time and treated with 2% Na_2CO_3 solution. Afterwards, the extract was treated with 1 gr Na_2SO_4 and was filtered with Whatman ® filter paper and the addition of 0.25 mL acetic acid onto obtained extract solution, it was filtered with 0.45 µm disc filter and after the evaporation step, dissolution which was rebuilt with 500 µL acidified water, applied to the HPLC as 20 µL. The separation was carried out by the C_{18} column (4.6×250 mm, 5 µm i.d.) and quantitated with diode array detector (DAD) set at 276 nm. Recovery was ≥94.9%. Limit of quantification (LOQ) was 0.6 ng/mL for patulin and 0.3 µg/mL for hydroxymethylfurfural HMF. The average level of patulin and hydroxymethylfurfural concentration were determined as 38.24 ng/mL±39.64 and 7.24 µg/mL±7.68 in all samples. Also, unpermitted concentrations of patulin and hydroxymethylfurfural in the samples were detected as 27.9% and 16.7%, respectively. The detected concentration of PAT was higher in the fruit juices ($p<0.001$) than baby food samples.



INTRODUCTION

Mycotoxins are produced by fungi as toxic secondary metabolites. These are low-molecular weight toxic chemical compounds with low volatility and are produced by certain filamentous fungi that colonize crops, in the field or post-harvest. Due to the ability to cause serious disease and death in animals and humans through the ingestion of contaminated food products, they have a great importance toxicologically.[1] Despite decades of extensive research, mold infection is still a challenging problem.[2] For this reason, the monitoring of these metabolites in food and drink has great importance, since they have lots of serious toxic effects on human health.

Patulin (PAT), 4-hydroxy-4H-fural[3,2-c]pyran-2(6H)-one, (Figure 1a) one of these mycotoxins, chemically defined as an unsaturated heterocyclic lactone. PAT is a polar molecule and has a relatively low molecular mass (154.12 gr/mol). PAT is stable in acid and has heat resistant properties.[3] Although patulin is non-stable in wet cereals, it is stable in dry cereals and during the production of cider.[13] It is produced by the fungal species which are *Byssochlamys*, *Aspergillus, Eupenicillium, Paecilomyces* and *Penicillium* growing on fruit.[4,5] Although PAT has been mainly found in apples and apple

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products it has also been identified in different types of fruit.[6] PAT is a natural contaminant, which is acutely toxic,[3,7] carcinogenic, teratogenic and is also a mutagenic metabolite.[8-10] It has been reported to cause immunotoxic, genotoxic, neurotoxic, embryotoxic[11] and gastrointestinal effects in rodents.[12]

PAT was tested as an antibiotic and antifungal in the past as both a throat and nose spray for treating the common cold and as an ointment for fungal skin infections. However, due to its severe median lethal dose, (LD_{50}) levels from 8 mg/kg (in rats) to 30 mg/kg bw (orl.ham), this practice has been abandoned and PAT was reclassified as a mycotoxin.[14,15] It has a broad toxicity spectrum of bacteria, fungi, plants, protozoa and animals.[12,16]

The International Agency for Research on Cancer (IARC) has classified PAT in category 3.[17] The provisional maximum tolerable daily intake (PMTDI) for PAT is established at 0.4 mg/kg body weight/day by The Joint Food and Agriculture Organization Expert Committee on Food Additives (JECFA).[6] PAT has been listed as a "**potential carcinogen**" from the office of Environmental Health Hazards Assessment in the USA. The WHO-recommended maximum levels of PAT are 50 ng/mL in apple juice, fruit nectars, reconstituted fruit juices, spirit drinks, cider and other fermented drinks derived from apple or containing apple juice. The maximum levels in solid apple products is 25 ng/g, and 10 ng/g in products for infants and young children.[18]

Due to the polar properties and low molecular mass of PAT, it is only retained on reverse-phase HPLC columns which use common mobile phases with the highest level of polar ingredients, *i.e.* mixtures of water and acetonitrile (up to 10%) or water and tetrahydrofuran (up to 5%).[19]

Hydroxymethylfurfural (HMF) (Figure 1b) is formed during the thermal treatment of foods containing carbohydrates particularly under acidic and high temperature conditions.[20] It is formed as a result of dehydration of ketopentoses. It is generated by acid-catalysed thermal dehydration from fructose, saccharose and glucose using the Maillard reaction. While, HMF is almost completely absent in fresh foods, depending on production technology and storage, it has been found in dried fruit, coffee, honey, UHT milk and caramel products. However, its levels in food are highly variable. It was detected in parenteral nutrient solutions which have glucose/fructose that were heat-sterilized. In addition to being used as a flavouring agent in food, it is also present in wood smoke and liquid smoke.[21,22] HMF concentrations contained in the food are used as an indicator of the heat and storage changes that they are exposed to during the preparation process and storing conditions. The presence of HMF in foodstuff is considered as an indication of deterioration. Although it is not clear whether HMF causes a potential health risk, some studies showed that high concentrations of HMF, are cytotoxic and can cause irritation to eyes, the upper respiratory tract, skin and the mucous membrane.[23] Oral LD_{50} was determined as 3.1 g/kg of body mass for rats.[24] If a product has a HMF-content of more than 5 mg/L in fruit juices, this indicates that a loss of quality and a HMF-content of more than 10 mg/L demonstrates unsuitable working technologies or extended storage in high temperatures. The recommended HMF concentrations by The International Federation of Fruit Juice Processors (IFFJP) are 5-10 mg/L and 25 mg/kg in fruit juice and fruit concentrates, respectively. A HMF concentration of 20 mg/kg was set as the limit for children by The European Union for juices.[25]

It is largely accepted that the presence of HMF and PAT are important quality criteria for many kinds of foods. Due to their similar chemical structure and properties, PAT and HMF have similar chromatographic properties. HMF is observed as a major interference during the liquid chromatographic analysis of PAT.[26]

Although there are a lot of instrumental analysis methods published for independent determination of PAT and HMF, there are several methods for the simultaneous analysis of these two analytes. These simultaneous methods are based on gas chromatography-mass spectrometry[27] and high performance liquid chromatography.[28,29]

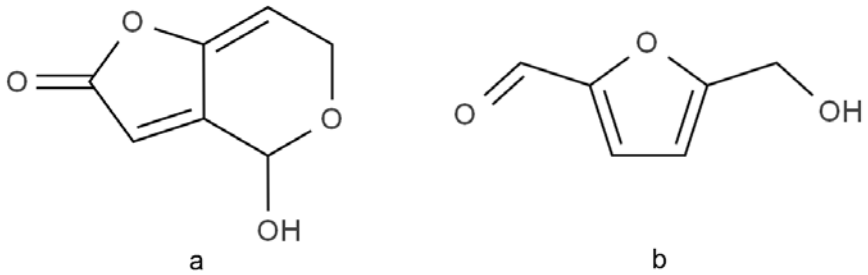

Fig. 1 – The chemical structures of PAT (a) and HMF (b).

In this study, the aim was to develop a rapid, relatively simple, reliable determination method for HMF and PAT and to validation to it extensively according to International Conference on[30] guideline and also investigate of PAT and HMF levels in fruit juices and baby food products were produced in Turkey.

RESULTS AND DISCUSSION

Linearity studies: The correlation coefficient (R^2) of the calibration curves for PAT and HMF were calculated as 0.9997 and 0.9995, respectively. Linearity tests were performed to cover the officially authorized and non-permissible levels of PAT and HMF. The linear range demonstrated a positive effect to this method, because the samples results showed considerable variation.

Sensitivity studies: The results of LOD and LOQ, which were obtained by the measurement of 10 individual quality control (QC) samples, demonstrated in Table 1. The LOQ values were calculated as 0.6 ng/mL for PAT and 0.3 µg/mL for HMF. It has been shown that the sensitivity values of the method are sufficient for the results obtained from the real samples (Figure 2c-d). PAT LOD result was 0.2 ng/mL that was better result when compared to previous studies.[29,31-33] However, the calibration range used for the HMF was determined with a narrow scope of accuracy for the study. The LOD of HMF was 0.1 µg/mL. The linear range of HMF was sufficient to evaluate the results obtained, and as a result, we had no samples below the calibration range.

Recovery studies: The results of recovery test for PAT were calculated as an average of 85.1 – 113.2% (98.7%). Also, recovery test results of HMF were obtained at 1, 5, and 25 µg/mL giving results of 82.3 and 106.3% (94.9%). Recovery results are displayed in Table 1. The recovery values demonstrated that this method was able to measure PAT and HMF in the samples with a high yield. It was observed that the extraction procedure was not complicated and did not require sophisticated instruments or complex application. Recovery values were obtained in the extraction procedure and demonstrated efficiency and reproducibility (Figure 2c-d).

Robustness studies: There weren't any significant changes observed in the analytical signals upon changing ultraviolet wavelength value (± 2 nm), mobile phase flow rate (± 0.1 mL/min), mobile phase organic solvent ingredient (± 4%), and column temperature (± 4 °C). In addition, the change of analysts and brand of solvents did not lead to significant changes in chromatographic signals or results.

Table 1

Linearity, sensitivity and recovery test results

	HMF	**PAT**
Retention time (min)	10.5	14.5
Linearity range (µg/mL) (n=5)	1.0–25.0	0.010–0.200
Slope	157693.0	139.9
Intercept	11701.0	1038.3
Coefficient of correlation, (R^2)	0.9995	0.9997
Detection limit (LOD) (µg/mL)	0.1 µg/mL	0.2 ng/mL
Quantification limit (LOQ) (µg/mL)	0.3 µg/mL	0.6 ng/mL
Recovery (low concentration)	82.3% (1 µg/mL)	85.1% (10 ng/mL)
Recovery (middle concentration)	96.2% (5 µg/mL)	97.8% (50 ng/mL)
Recovery (higher concentration)	106.3% (25 µg/mL)	113.2% (200 ng/mL)

Stability studies: The stability of PAT (10, 50 and 200 ng/mL) and HMF (1, 5 and 25 µg/mL) in the stock solutions were assessed under several conditions. The stability of stock solutions at room temperature were evaluated at 1, 2, 3 and 4 week periods. The freeze-thaw stability test was executed by three QC samples after conducting five repeated freeze-thaw stages. The long-term stability test was carried out for 1, 2 and 3 months using QC samples maintained at -18 °C. There was no significant decrease or degradation observed in the concentration of PAT and HMF in three different tıme perıods. The relative standard deviation in all stability test samples was less than 4.3 RSD%.

Sample analysis and results

After the analysis methods were established, optimized and valuable validation results were obtained, the 86 apple-based samples including fruit juice (n=68) and fruity baby food samples, (n=18) were successfully analysed (Figure 2c-d). The real fruity samples results were successful in concordance with the results obtained from validation tests. The analyses of all samples were carried out in isocratic conditions, with less than 16 minutes in the chromatograms without any shift, interference and carryover problems. During the analysis of the real samples, no problem or pressure changes were observed that could have a negative effect on the analytical column. Therefore, there was no need for a guard column. It is thought that the addition of Na_2CO_3 and Na_2SO_4, especially for the cleaning of phenolic substances and polar content, has a positive contribution to the sensitivity, recovery and selectivity of the study. It was found in the chromatograms that the cleaning application of the samples with 0.45 µL Whatman® filter paper and 0.45 µL disc filter contributed positively to the study. At the same time, it was observed that these application steps did not have a negative effect on recovery.

In addition, the liquid obtained during the evaporation of nitrogen and subsequent reconstitution of the residue (0.50 mL of acidified water) had very striking colours. Therefore, it is suspected that food colours were added to the content of fruit juices and fruity baby foods. However, mainly yellow, orange, purple, red and green coloured residues did not show any adverse effect on the chromatograms. The robustness of the method, in agreement with the other tests obtained from validation tests, had a positive effect on the sample results.

In addition, it was important to select fruit juices and apple-based baby foods that were considered healthier than carbonated drinks in the sample analysis of the study. Another aim of this study was to determine the risk of exposure to PAT and HMF in specific groups. Therefore, analyzes were carried out on baby foods and fruit juices. For the reliability of the results obtained from the study, samples remaining 3 months or less before the end of their shelf life were excluded from the analysis due to the risk of contamination with mycotoxin.. If the shelf life is uncertain, the product is discolored or the container is damaged, the samples were not analyzed for reliability due to the false-positive risk for HMF and patulin. In addition to that, at the stage of collecting samples from the markets, care was taken to select products that represent the preferences of all segments of the society.

No complex devices and materials were needed in the sample preparation and extraction process. Although a significant number of samples were analysed (n=86), the time and labour needed in the sample preparation phase was given approval for routine analyses. The lowest volume of sample and the solvent (ethyl acetate) required for the efficient extraction was 5 mL. Analysis costs showed that the method could be evaluated as an economic analysis method.

Considering the average HMF and PAT levels of all samples, it was observed that the samples contained higher levels of HMF when compared to PAT (Table 2 and 3). The mean HMF in all samples was 7.24 µg/mL ± 7.68 (mean ± SD). HMF was detected, with an average value of 5.95 µg/mL ± 7.36 (mean ± standard deviation) (Table 4). In 56 of the samples analysed HMF values (11.69 µg/mL ± 7.27) (mean ± SD) were found to be approximately two fold higher in fruit juices (n = 68), however this result was not statistically significant (Table 4). It is known that the high sugar content in the sample is an important factor in HMF formation. As a parallel to the higher rate of carbohydrates in fruit juices compared to commercial fruity baby food samples, the HMF results observed were expected. However, the observed results were between 0.60 and 28.26 µg/mL (mean 5.95 µg/mL) which is important as it reflects the risk of exposure to a secondary compound suspected to be toxicologically risky. The results are very important in terms of the exposure risk of these products known to be consumed by a large part of the population.

Table 2

HMF and PAT levels determined in fruit juice samples

Sample No	HMF (µg/mL)	PAT (ng/mL)	Sample No	HMF (µg/mL)	PAT (ng/mL)	Sample No	HMF (µg/mL)	PAT (ng/mL)	Sample No	HMF (µg/mL)	PAT (ng/mL)
1	28.26	10.61	18	2.30	21.57	35	7.25	111.32	52	ND	9.57
2	2.52	12.55	19	1.40	87.05	36	3.36	62.41	53	1.43	48.55
3	6.00	18.36	20	ND	9.21	37	2.89	71.52	54	27.73	17.22
4	2.16	17.60	21	0.71	22.76	38	2.82	69.22	55	3.99	24.89
5	4.87	16.52	22	5.15	23.33	39	4.08	128.83	56	9.88	47.13
6	1.42	14.50	23	1.04	39.51	40	ND	31.88	57	23.71	ND
7	3.07	ND	24	25.30	118.15	41	1.51	15.35	58	2.39	18.49
8	5.85	ND	25	1.70	67.52	42	ND	ND	59	6.36	16.24
9	3.58	ND	26	3.48	87.51	43	2.72	28.93	60	1.59	31.45
10	1.88	16.42	27	1.20	19.13	44	23.31	112.52	61	5.44	10.45
11	0.98	ND	28	5.95	79.25	45	2.90	44.91	62	3.42	9.21
12	1.11	12.86	29	0.68	19.55	46	ND	13.64	63	9.86	30.08
13	1.12	44.80	30	0.69	30.52	47	4.98	84.33	64	0.60	16.55
14	1.74	40.86	31	2.58	23.07	48	8.13	37.01	65	ND	28.31
15	2.18	111.95	32	2.75	76.17	49	25.88	188.86	66	3.65	27.03
16	6.30	16.17	33	3.78	141.47	50	1.99	63.61	67	6.70	40.51
17	14.17	44.05	34	20.96	153.95	51	2.28	24.60	68	1.41	67.13

Table 3

HMF and PAT levels determined commercial fruity baby food samples

Sample No	HMF (µg/mL)	PAT (ng/mL)	Sample No	HMF (µg/mL)	PAT (ng/mL)
1	7.98	4.11	10	2.80	3.18
2	15.90	6.47	11	20.63	5.33
3	16.35	4.95	12	17.85	4.16
4	3.80	3.07	13	5.07	8.95
5	18.18	6.25	14	9.22	11.71
6	2.75	13.83	15	15.90	16.60
7	16.05	6.14	16	2.97	8.31
8	4.88	7.20	17	19.71	7.72
9	24.32	4.45	18	6.04	8.19

Table 4

Descriptive statistics obtained from two sample groups

	Fruity baby food products		Fruit juice samples	
	HMF (µg/mL)	PAT (ng/mL)	HMF (µg/mL)	PAT (ng/mL)
Mean	11.69	7.25	5.95	47.24
SD	7.27	3.67	7.36	40.81
RSD%	62.23	50.57	123.62	86.40

In a study, conducted by Vorlova *et al.* (2006), HMF levels were found to be between 2.1 and 9.8 mg/kg (mean: 7.15 mg/kg) in the infant formula samples (n=12). In the same study, HMF levels in fruit juices (n=12) were observed to be between 0.00 and 2.8 mg/kg (0.4 mg/kg ± 0.75; X ± SD).[34] Matic *et al.* (2009), detected HMF levels to be 9.89 mg/kg ± 12.1 in fruit juice samples (n = 20) in their study. Also, three of the samples have a higher HMF concentration than suggested, which has reflected the maximum prescribed value of HMF.[24]

In this study with a total of 84 samples, PAT was detected in all samples, except 6 (Table 2). The PAT levels in the samples were between 3.07 and 188.86 ng / mL (38.24 ng / mL ± 39.64) (mean ± SD). The PAT levels in fruit juice samples (n=68) were between 9.21 and 188.86 ng / mL (47.24 ng / mL ± 40.81) (mean ± SD). In the fruity baby food samples, PAT levels were found between 3.07 and 16.60 ng/mL (7.25 ± 3.67 ng/mL, mean ± SD) (Table 4).

In this study, the amount of patulin in fruit juice samples (Table 2) was significantly higher than the amount of patulin in fruity baby food samples (Table 3) ($p < 0.001$). Al-Hazmi (2010) investigated patulin levels with another mycotoxin in 51 fruit juice samples from 17 groups in his study.[35] The PAT was detected as a concentration of 151.5 ng/mL in only one type out of 17 in total (5.88%). It was observed that this result was approximately 3 times higher than the 50 ng/mL allowed by the World Health Organization. In the study conducted by Lai *et al.* (2000), patulin levels were determined in 105 apple juice and apple based mixed fruit juices.[3] Patulin was detected in 12 of the (11.4%) samples. The detected concentrations are between 15.4 and 39.9 ng/mL and all of the observed samples have patulin values below those suggested by the World Health Organization. Karaköse *et al.* (2014) published a study in 2014

which showed that the amount of patulin in the samples analyzed was less than 10 ng/mL.[32] Aktas *et al.* (2004), found approximately 70 ng/mL patulin in one group (25%) out of 4 research groups.[19] Boonzaaijer *et al.* (2005) investigated the patulin levels of 63 commercial apple products[36] and patulin was detected in only 1 (1.59%) of 63 samples. In the study which was conducted Gökmen and Acar (1998), 215 fruit juice samples were analysed, and it was detected that PAT concentrations in the samples ranging from 7 to 376 ng/mL.[28] It was found that 43.5% samples have higher patulin concentrations. Although the results of PAT and HMF obtained in our study were compatible with the literature, the amount of PAT and HMF were found to be significantly higher than the permissible levels.

Statistical analysis: All statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) 22.0. Statistical analysis was performed using Student's t test for two independent means, with a p value <0.05 considered to be statistically significant.

The author of this paper has not a financial or personal relationship with any organization that may inappropriately affect or bias the contents of the paper.

EXPERIMENTAL

Chemicals

The HPLC grades chemical standards of PAT (with purity of ≥ 99%) (Figure 1a) and HMF (Figure 1b (HPLC grade) which were purchased from Sigma-Aldrich (Missouri, USA). HPLC grade acetonitrile, ethyl acetate, glacial acetic acid, sodium carbonate, and anhydrous sodium sulphate (analytical grade) were ordered from Sigma-Aldrich (Missouri, USA). PTFE membrane filter (0.45 µm pore size, 47 mm diameter) purchased from Millipore (Massachusetts, USA) and disc filter (0.45 µm pore size) was obtained from (Alltech). Ultrapure water was obtained from the Elga Purelab Water Purification System (Buckinghamshire, UK). The quantitative filter paper was purchased as Whatman No. 40.

Instrumentation

The separation and quantification were performed by Shimadzu LC-20AD high-performance liquid chromatography (HPLC) system (Kyoto, Japan) equipped with a degasser (DGU-20A5R), an automatic liquid sampler (SIL-20ACHT) which has a 20 µL sample loop, a column oven (CTO-20AC) and a diode array detector (DAD) (SPD-M20A). Analytical separations were carried out with a reverse phase C_{18} analytical column (250 x 4.6 mm i.d., 5 µm particle size) obtained from the ACE-5 (Scotland, UK). The analysis was carried out under isocratic conditions using a flow rate of 1.0 mL/min at 30°C. Chromatographic determinations were performed at 276 nm. The mobile phase consisted of acetonitrile and water (5:95, v/v) and it was degassed in the ultrasonic water bath 30 minutes before every use. Optimum analytical conditions were set following an optimization procedure for column selection, mobile phase content and wavelength. The DAD detector was fixed at 276 nm for determination of PAT and HMF. The best separation was obtained from RP C_{18} ACE-5 analytical column which was filled with 5 µm ODS column filling material.

Stock Solutions and Working Standards

The main stock solution of PAT (1 mg/mL) was prepared in methanol and stored at -18°C during use. The main stock solution of HMF (20 mg/mL) was prepared in methanol and stored at -18°C during use. Intermediate stock and working solutions were prepared daily from the main stock solution with acidified water as the solvent (pH 4). It was observed that the PAT and HMF main stock solutions were chemically stable at +4 °C for at least 1 month. Na_2CO_3 (2% w/v) solution was formed by dissolving 10 g of sodium carbonate in 500 mL of distilled water and it was stable at +4 °C for a minimum of 1 month.

Sample Collection

Samples were purchased from retail markets and they were stored at +4 °C until analysis. Products with apple and apple based ingredients were selected. As a rule, PAT and HMF levels in fruit juices and commercial fruity baby food samples were measured in less than one month. Samples were produced by 12 different Turkish manufacturers. It is possible to assume that the analysed samples in this study represent the basis of samples sold in the middle of Turkey.

Sample Preparation

5 mL sample was extracted and added to (10 mL) ethyl acetate and was spun twice by a rotator mixer at 800 rpm, in 10 min. The organic phases were separated in each step and collected in 50 mL falcon tube. In order to remove phenolic acid, the acetate fractions were treated with 2 mL Na_2CO_3 solution (2% w/v). Since PAT and HMF are unstable in alkaline conditions, this step was completed in exactly in 3 minutes. The residue was re-extracted with 5 mL more ethyl acetate. Afterwards, 1 gr Na_2SO_4 was added into the ethyl acetate extract to the collect of all the remaining polar phases, and it was extracted by the rotatory mixer at 800 rpm for 5 mins. All ethyl acetate extracts were filtered with the Whatman® filter paper and thereafter 2 mL of ethyl acetate was added to wash the filter cake layer and all extraction solutions were merged and then 0.25 mL acetic acid was added into the extract for acidification. Extract (approximately 27 mL volume) was filtered with 0.45 µm disc filter. Finally, the extract was evaporated in 30 min under the gentle and constant flow of nitrogen. The residue was rebuilt in 500 µL acidified water (pH 4.0) and then immediately injected to the HPLC as 20 µL.

Method Validation

This method was validated according to the specificity, selectivity, linearity, sensitivity, recovery and robustness. The validation protocol was applied considering reproducibility of method and instrument to obtain accurate and precise measurements in agreement with ICH guidelines.[30]

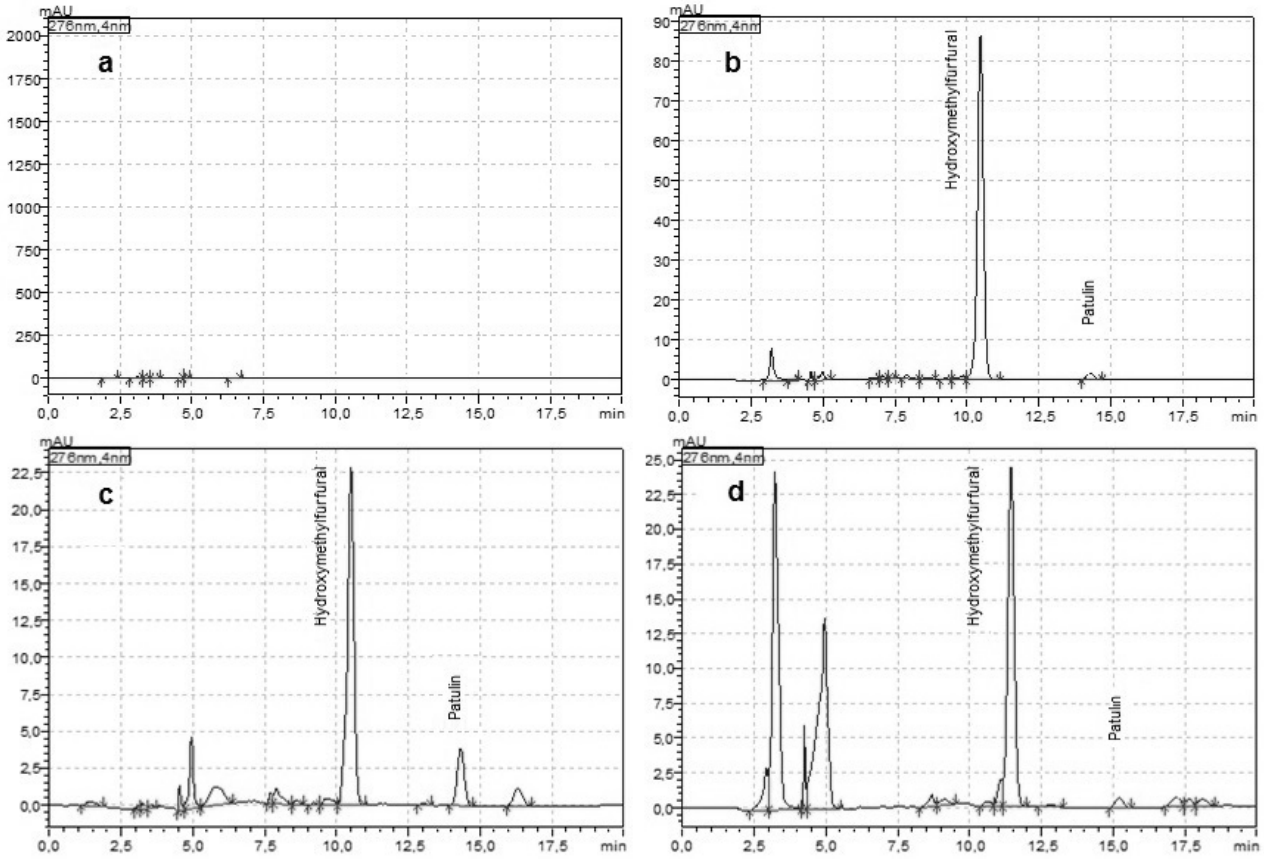

Fig. 2a – A blank chromatogram sample, 2b. A sample chromatogram of HMF and PAT standards (25 µg/mL and 20 ng/mL, respectively) that were used as a quality control sample in the study 2c. The sample chromatogram obtained from a commercial fruity baby food product extract, 2d. A sample chromatogram which belongs to an apple juice sample.

Table 5

Robustness data of the described method representing as the RSD% value

Analytes	**Mobile phases solvent content (± 4%)**	**Ultraviolet wavelength (± 2 nm)**	**Flow rate (± 0.1 mL/min)**	**Column temperature (± 4 °C)**
PAT (50 ng/mL)	4.83	3.21	2.44	2.72
HMF (5 µg/mL)	3.52	2.87	2.16	2.10

Specificity and Selectivity: The method showed excellent chromatographic specificity without any endogenous interference at the retention times of HMF and PAT (10.5 and 14.5 min) in not only quality control samples but also real commercial fruity baby food products and fruit juice samples. Representative chromatograms, which are blank (Figure 2a) and spiked (Figure 2b) illustrate the high chromatographic resolution that conducted in less than 16 minutes.

Linearity: After chromatographic conditions were established and optimized, matrix-based calibration curves were plotted for PAT and HMF. Curves were created with standard addition method and each of the calibration points were determined by independent samples (n=3). The calibration curve of PAT was prepared with 10, 25, 50, 100 and 200 ng/mL concentrations. Also, HMF calibration curve was prepared with 1.0, 2.5, 5.0, 10.0, and 25.0 µg/mL concentrations.

Sensitivity: The limit of detection (LOD) and limit of quantification (LOQ) were determined according to the ICH recommendations based on the standard deviation of the response and the slopes of the calibration graphs. LOD= 3.3σ/S; LOQ= 10σ/S (σ: The standard deviation of the response; S: The slope of the calibration curve). The concentrations of 10 ng/mL PAT and 1 µg/mL HMF were used to the calculation of LOD and LOQ levels, respectively.

Recovery: The recovery of extraction was determined by comparing pre-extraction spikes with the post-extraction spiked analytes. Five individual replicates of spiked samples at

low, middle and high concentrations of PAT (10, 50 and, 200 ng/mL) and HMF (1, 5 and, 25 μg/mL) were prepared. The extraction procedure was carried out as described before the sample preparation step.

Robustness: Mobile phase solvent content (± 4%), ultraviolet wavelength (± 2 nm), mobile phase flow rate (± 0.1 mL/min), and column temperature (± 4 °C) changes were all examined.

CONCLUSIONS

The developed method was successfully applied to 18 fruity baby food samples with 68 fruit juices. The liquid-liquid extraction method yielded a high recovery of 94.9% and 98.7% for PAT and HMF, respectively. This extraction method doesn't need any complicated instrument and it is relatively simple for routine analysis. The linearity results was excellent with a value of ≥0.9995. Total analysis run time was 16 min which is suitable for routine analysis of HMF and PAT. Results for both PAT (LOD: 0.2 ng/mL) and HMF analysis displayed high sensitivity. Methods did not show any changes due to modifications of the mobile phase organic solvent (± 4%), column oven temperature (± 4°C), mobile phase flow rate (± 0.1 mL/min) and ultraviolet wavelength (± 2 nm). The stability test of stock solutions were applied at different conditions which allowed a maximum of five repeated freeze-thaw periods in 3 months. The relative standard deviation of the stability test results were less than 4.3%. As this analysis method does not require a sophisticated instrument for chromatographic determination, it can be applied routinely to analyse HMF and PAT in toxicological reference laboratories, food analysis and control laboratories.

The use of this method for the analysis of 86 samples showed both the applicability of the method and the monitoring of patulin and HMF levels in fruit juice and infant formula samples. The results are noteworthy for both HMF and patulin, However patulin displayed toxicological significance. Patulin was detected in 62 of 68 fruit juice samples and in all 18 fruity baby food samples. A high amount of patulin was determined in 16.67% of infant formula and 27.94% of fruit juices. In 11.11% of infant formulas and 10.29% of fruit juice samples, a high amount of HMF was detected.

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