Optimization and applicability of high performance liquid chromatographic methods for quantification of patulin and ochratoxin A in fruit purées

ELENA BELAJOVÁ

Summary

Patulin and ochratoxin A are mycotoxins generated in apples by certain strains of fungi *Penicillium* spp. and *Aspergillus* spp. Present work was aimed at occurrence of these mycotoxins in processed fruit-based and fruit-cereal mixed purées for infants and small children. For the purpose, two independent methods were optimized and verified. The methods were based on high performance liquid chromatography using diode-array detection and fluorescence detection and provided quantitative data on patulin and ochratoxin A contents, respectively. For sample pre-treatment, conventional liquid-liquid extraction was replaced with simpler procedures followed by solid phase extraction. The limit of detection was 5.8 $\mu g \cdot kg^{-1}$ for patulin and 0.06 $\mu g \cdot kg^{-1}$ for ochratoxin A, the limit of quantification was 6.9 $\mu g \cdot kg^{-1}$ and 0.12 $\mu g \cdot kg^{-1}$, respectively. The recoveries varied in the range of 79–92 % for patulin and 78–105 % for ochratoxin A at defined spiking levels. The described methods were applied in analyses of 114 single- and multi-component purée samples, the results showing low-percent incidence of patulin and ochratoxin A (5.6 % and 8.0 %, respectively). Positive samples were contaminated with mycotoxins at content levels not exceeding the EU limit. Simultaneous incidence of the two mycotoxins was not confirmed in any purée sample.

Keywords

patulin; ochratoxin A; high performance liquid chromatography; fruit purée

Mycotoxins contaminate cereals and cereal-based products in general, however, growth of toxinogenic fungi is also observed on some fruits. The most widespread mycotoxins in fruits and fruit products are patulin (PAT), ochratoxin A (OTA), aflatoxins and *Alternaria* toxins [1–3]. Processing of fruits, in particular of apples, is widely related to contamination with PAT. Besides apples, PAT was detected in grapes, pears, peaches, apricots, strawberries, blueberries [4] and dried figs [5]. PAT is produced by fungi *Penicillium* spp., *Aspergillus* spp. and *Byssochlamys* spp., of which the most expansive is the species *Penicillium expansum,* known as blue rot mould. This fungus is capable of producing 800–12 500 μg of PAT per 1 kg of rotten apple tissue [6]. PAT can survive thermal treatment in food processing [7] as well as the temperature of 0 °C [6]. With respect to carcinogenic effects of PAT to humans, International Agency for Research on Cancer (IARC) has classified PAT as Group 3 carcinogen (not classifiable as to its carcinogenicity to humans) [8].

The toxic effect of PAT can elevate other mycotoxins being present in apple products, for example OTA. OTA is a nefrotoxic toxin damaging kidneys and urinary tract [9], classified as group 2B carcinogen by IARC (i.e. as possibly carcinogenic) [8]. OTA is generated by fungi *Aspergillus ochraceus, A. carbonarius* and *Penicillium verrucosum* [9]. Analogous to PAT, OTA is relatively stable at high temperatures and only partially degrades in fermentation. OTA occurs mainly in wines and has not been identified in apple juice so far, even though it is produced by the same fungi as PAT [10]. Previous investigations showed that apples may contain OTA-producing moulds not always capable of producing OTA [11].

Within the context of mycotoxins' toxicity, infants and small children are considered as the most at risk as they are low in their body weight and

Elena Belajová, Department of Chemistry and Food Analysis, Food Research Institute, National Agricultural and Food Centre, Priemyselná 4, P. O. Box 25, SK-82475 Bratislava, Slovakia. Tel.: 00421 2 50237 051, fax: 00421 2 55571 417, e-mail: elena.belajova@nppc.sk

© 2020 National Agricultural and Food Centre (Slovakia)

may consume more frequently potentially contaminated food. Therefore, the maximum allowable limits are set stricter for mycotoxins in baby food. Codex Alimentarius Joint Expert Committee for Food Additives and Contaminants (JECFA) has implemented the Provisional Maximum Tolerable Daily Intake (PMTDI) of 0.4 $\mu g \cdot kg^{-1}$ body weight (bw) per day for PAT in apple juice and apple products for all age groups [12]. The maximum limit for PAT has been stated at 50 $\mu g \cdot kg^{-1}$ in apple juice, but since PAT is found in many types of fresh or processed fruits and vegetables, the regulatory level of 25 $\mu g \cdot kg^{-1}$ for solid products and 10 $\mu g \cdot kg^{-1}$ for food for infants and young children has been set [13]. Regarding OTA, the European Commission has drafted the OTA level of 0.5 $\mu g \cdot kg^{-1}$ for baby food and processed cereal-based food for infants and young children [13].

PAT and OTA incidence in baby food was widely reported in recent years, including cereals and cereal-based products, but only limited information is available on fruit- and vegetable-based products for babies such as apple purées or other processed fruit-based products. Therefore, the purpose of this work was to elaborate two independent liquid chromatographic methods with diode-array and fluorescence detection for PAT and OTA determination, respectively, in order to monitor their presence in apple-based products intended for infants and small children. The outcomes of study originate from the several years' monitoring programme in Slovakia.

MATERIAL AND METHODS

Material

Reference materials of OTA (99 %), PAT (99.7 %) and 5-hydroxymethylfurfural (HMF) (99 %) were obtained from Sigma-Aldrich (St. Louis, Missouri, USA). Pectinase enzyme solution (activity 1400 $U \cdot g^{-1}$) was obtained from R-Biopharm Rhône (Glasgow, United Kingdom). All used chemicals were of analytical purity or purity grade "for high performance liquid chromatography" (HPLC). Acetonitrile (ACN) and methanol (MeOH) Chromasolv were from Sigma-Aldrich; glacial acetic acid (99.6 %) and toluene were from AFT (Bratislava, Slovakia); ethyl acetate was from Centralchem (Bratislava, Slovakia); diethyl ether was from Mikrochem (Pezinok, Slovakia); potassium dihydrogen phosphate, potassium chloride, sodium chloride and hydrochloric acid (35 %) were from Lachema (Brno, Czech Republic); sodium hydrogen carbonate was from Slavus (Bratislava, Slovakia); absolute ethanol (99.7 %) and disodium hydrogen phosphate were from Merck (Darmstadt, Germany); compressed nitrogen gas (technical purity 4.0) was from Siad Slovakia (Bratislava, Slovakia).

Equipment

For the standard solutions and sample preparation, the following equipment was used: ultra violet visible near infrared (UV-VIS-NIR) spectrophotometer UV 3600 (Shimadzu, Kyoto, Japan); analytical scales Mettler AE 200 (Mettler Toledo, Columbus, Ohio, USA); microprocessor pH meter HI 223 with glass body combination pH electrode HI 1131P (Hanna Instruments, Woonsocket, Rhode Island, USA); orbital shaker OS-20 (Biosan, Riga, Latvia); ultrasonic bath UC 005 AJ1 (Tesla, Praha-Strašnice, Czech Republic); oil suction pump (Reglerwerk, Dresden, Germany); 12-port solid phase extraction (SPE) vacuum manifold (Phenomenex, Torrance, California, USA); centrifuge 2-16KC (Sigma Aldrich); air dryer KCW-100 (Premed, Marki, Poland); device for preparation of deionized water Rodem 6 (Ecotest, Topoľčany, Slovakia); immunoaffinity columns (IAC) Ochraprep and molecularly imprinted columns (MIP) EASIMIP Patulin (R-Biopharm Rhône); SPE columns Oasis HLB (3 ml/60 mg; Waters, Milford, Massachusetts, USA); syringe filters of 0.45 μm pore size with methylcellulose membrane and a fast filtration paper KA 3, 150 mm diameter (Fisher Scientific, Hampton, New Hampshire, USA).

A liquid chromatograph system Agilent Technologies 1100 Series (Agilent Technologies, Santa Clara, California, USA) consisted of a quartenary pump, degasser, auto-sampler, diode array detector (DAD) and fluorescence detector (FLD). The tested columns for PAT analysis were Column 1: Zorbax SB-C18, 250 mm × 4.6 mm, 5 μm particle size (Agilent Technologies); Column 2: monolithic column Chromolith Performance RP-18e, 100 mm × 4.6 mm (Merck); Column 3: Purospher STAR RP-18e, 250 mm × 4.6 mm, 5 μm particle size (Merck). For OTA analysis, only Column 1 was used.

Preparation of standard solutions

Stock solution of PAT (concentration of 200 $\mu g \cdot ml^{-1}$) and PAT stock calibration solution (concentration of 10 $\mu g \cdot ml^{-1}$) were prepared in accordance with MACDONALD et al. [14]. The stock solution was stored frozen at –20 °C for a maximum of 3 years until restored with a new PAT reference material. During this time, the mass concentration (in micrograms per gram) of the PAT stock solution was determined minimally

twice in order to check stability of the solution. The stock calibration solution of PAT was prepared as follows: the volume of PAT stock solution, which contained exactly 10 μg of PAT, was evaporated under nitrogen stream and the residue was dissolved in 1 ml of 0.1 % acetic acid. The working calibration solutions were prepared from the stock calibration solution by its dilution with 0.1 % acetic acid in the range of concentrations 12.5–500 ng·ml^{-1} (corresponding to contents of 5.7–188.5 μg·kg^{-1}). The working calibration solutions were prepared freshly on the day of use.

The OTA stock solution (concentration 1.25 mg·ml^{-1}) was prepared by dissolving 5 mg of crystalline OTA in 4 ml of the mixture toluene-acetic acid in a ratio of 99 : 1 (v/v). The stock calibration solution (0.125 mg·ml^{-1}) was prepared as follows: 0.5 ml of OTA stock solution was evaporated under nitrogen stream and residue was dissolved in 5 ml of the mobile phase for OTA determination (deionized water-ACN-acetic acid, 49.5 : 49.5 : 1 (v/v/v)). The working calibration solutions were prepared freshly from the stock calibration solution by its dilution with OTA mobile phase in the range of 0.00013–1.25 μg·ml^{-1} (corresponding to contents of 0.03–312.50 μg·kg^{-1}). The storage conditions for OTA stock solution and OTA stock calibration solution corresponded to those of the PAT stock solution. The stability of OTA stock calibration solution was checked by HPLC analysis through peak area response.

Preparation of phosphate-buffered saline solution

Phosphate-buffered saline (PBS) necessary for OTA analysis was prepared by dissolving 8.0 g sodium chloride, 1.2 g disodium hydrogen phosphate. 0.2 g potassium dihydrogen phosphate and 0.2 g potassium chloride in 990 ml of deionized water. The pH value of the solution was adjusted to 7.4 with hydrochloric acid. The total volume of the solution was made up to 1000 ml.

Samples

All samples came directly from the production of a major Slovakian manufacturer of fruit- and vegetable-based baby food (Novofruct SK, Nové Zámky, Slovakia). Within the years 2013–2019, 89 single-component purée samples (based on apples or cherries) as well as multi-component samples based on apples, pears, banana, strawberries, blueberries, plums, apricots, cranberries, mango and peach were analysed. Further, 25 purée samples based on apples, other fruits and cereals or pseudo-cereals, such as wheat, buckwheat, oat or millet flakes, cultivated flax, and quinoa, were tested for OTA. The purée samples (ready to be placed on market) were delivered in original commercial packages, i.e. in sterile glass bottles or food grade torch packages, which were stored at laboratory temperature for maximum 10 days. In case of intermediary products, single-component purées in a non-commercial package (ready to further processing), these were stored at 5–7 °C for maximum 5 days.

Preparation of samples for patulin analysis

Two types of SPE column were tested that are recommended for clean-up and concentration of extracts with PAT content.

1. Solid phase extraction by EASIMIP Patulin

The first step in sample preparation procedure was incubation of the purée sample with pectinase. An amount of 10 g of purée was weighed into a plastic centrifugation tube and then 10 ml of water and 150 μl of pectinase solution were added. The stoppered tube was manually shaken for 20 s and then incubated at 40 °C for 2 h. Afterwards, the sample was centrifuged at 700 ×*g* for 20 min and the obtained supernatant was filtered through a fast speed paper filter. In next steps, the sample filtrate underwent cleaning on EASIMIP Patulin columns using SPE vacuum manifold. Each column was conditioned with 2 ml of 100% ACN and immediately afterwards with 1 ml of deionized water passing through the column at a flow rate of 1 drop per second. Then, 5 ml of the sample filtrate was loaded onto wet surface of the column's filling at a flow rate of 1 drop per 2 seconds and, afterwards, the columns were washed with 4 ml of 1% acetic acid and 4 ml of deionized water. Columns were dried with air to remove residual liquid and then, 0.5 ml of 100% diethyl ether was added and the columns were again dried with air. PAT was eluted with 2 ml of 100% ethyl acetate. Then, 10 μl of 100% acetic acid was added to the eluate and, after intensive mixing, the solvent was evaporated by nitrogen gas at 35–45 °C. Finally, the residue was dissolved in 0.5 ml of 0.1% acetic acid.

2. Solid phase extraction by Oasis HLB

Incubation of purée samples with pectinase was performed in the same manner as described above for Procedure 1. The procedure of PAT extraction described by EISELE and GIBSON [15] was performed with a modification, when an SPE multiport vacuum manifold was used instead of manual application of plastic syringes. Each SPE column was conditioned with 2 ml of deionized water, 2 ml of MeOH and again with 2 ml of deionized water. Then, 2.5 ml of the extract was loaded onto a wet

surface of the column's filling. After the sample had passed through, the column was washed with 2 ml of 1% sodium hydrogen carbonate solution followed by 2 ml of 1% acetic acid and then dried with air for 8–10 min. PAT was eluted with 1 ml of a mixture ethyl acetate-diethyl ether (1:9, v/v). The obtained eluate was dried by nitrogen stream and the residue was dissolved in 0.25 ml of 0.1% acetic acid.

Preparation of sample for ochratoxin A analysis

To 25 g of a purée sample in a conical flask, 100 ml of the mixture ACN-water (60:40, v/v) was added, vortex-mixed at approximately 3.0 Hz for 20 min and then ultrasonicated for 10 min. The sample was filtered through a fast filtration paper and then, a volume of 44 ml of PBS was added to 4 ml of the filtrate and intensively mixed. SPE-IAC columns Ochraprep were tempered to laboratory temperature during several hours and then conditioned with the PBS solution being present in the column. The entire volume of the diluted extract (48 ml) was applied onto IAC and let to pass through by gravity. The columns were washed with 20 ml of PBS and dried with air for 8–10 min. OTA was eluted with 2 ml of 100% MeOH. The obtained eluate was dried by nitrogen stream and the residue was dissolved in 0.25 ml of the mobile phase.

Final HPLC conditions

In PAT analysis, DAD was used at 276 nm. The mobile phase consisted of A: ACN-water (5:95, v/v) and B: 100% ACN. The following programme of gradient elution was used: 0–15 min: 100 % A – 0 % B, 15–18 min: 20 % A – 80 % B, 18–30 min: 20 % A – 80 % B, 30–32 min: 100 % A – 0 % B, 32–40 min: 100 % A – 0 % B. The flow rate of the mobile phase was 1 ml·min^{-1}, injection volume was 100 μl and separation column temperature was 30 °C. In OTA analysis, FLD excitation was set at 333 nm and emission wavelength was set at 460 nm. The mobile phase was ACN-acidified water (20 ml of acetic acid in 1 litre) in a ratio of 50:50 (v/v) at a flow rate of 1 ml·min^{-1}, injection volume was 100 μl and separation column temperature was 25 °C.

Identification of mycotoxins

Mycotoxins were identified by retention times and by spectral analysis, which comprised comparing the sample peak spectrum with that of pure standards by Agilent chromatographic software ChemStation (Agilent Technologies) and stored in a home spectra library (component part of ChemStation). The spectra of PAT scanned with DAD over the wavelength range of 190–400 nm were utilized for identification as well as for checking the peak purity of PAT in tested samples. For identification of OTA, the spectra in the excitation range 220–380 nm and emission range 300–500 nm were used.

Validation of methods

For validation of the described methods, calibration/linearity, limit of detection (*LOD*), limit of quantification (*LOQ*), precision, accuracy, uncertainty of the measurement (*U*), selectivity, and ruggedness were estimated. The calibration data acquired by analysis of six calibration solutions including blank sample were processed through the linear regression diagnostic by Excel XP software (Microsoft, Redmont, Washington, USA). *LOD* and *LOQ* were calculated on the basis of blank sample analysis according to the equations:

$$LOD = X_0 + 3s_x \quad (1)$$

$$LOQ = X_0 + 10s_x \quad (2)$$

in which X_0 is an average area and s_x is a standard deviation of an average area.

Precision was expressed as repeatability relative standard deviation (RSD_r) of the mean of five replicates of standard solutions (expressed in percent). Recovery rate (R) as a measure of accuracy (expressed in percent) was calculated from spiked purée sample at three different content levels, using the formula:

$$R = \left(\frac{C_S - C_{NS}}{C_A}\right) \times 100 \quad (3)$$

in which C_S is content of mycotoxin in spiked sample, C_{NS} is content of mycotoxin in unspiked sample and C_A is content of mycotoxin in the spiking solution. Content was expressed in micrograms per kilogram.

The extended uncertainty of the measurement (U), which equals $2 \cdot U_C$ with covering factor of 2, was an associated of type A (U_A) and type B (U_B) uncertainty, arising from extraction and analytical processes (not from sampling). Selectivity was determined by measuring resolution (R_S) of the mycotoxin peak from interfering peaks. Additionally, in PAT analysis, assessment using match factor spectral analysis was accomplished, in which an internal criterion for purity factor was set at ≥ 995. In the evaluation of ruggedness, some variations in methods' parameters were tested within a longer time period to define their susceptibility to outer changes (e.g. new lots of mycotoxin standard and solvents, fluctuation of ambient temperature, slight changes in analytical equipment stability).

The studied matrix for method validation was baby apple puree free from PAT (in PAT determination) and apple purée with pears and biscuits for babies, with natural OTA content of $0.17 \pm 0.09\ \mu g \cdot kg^{-1}$ (in OTA determination).

RESULTS AND DISCUSSION

Optimization of chromatographic separation

In this part of the study, a model solution of pure PAT and HMF without fruit matrix was used. Various procedures were tested for PAT determination with different mobile phase composition (ACN-water in ratios 1:99, 5:95, 20:80 (v/v); ACN-1% acetic acid in ratio 1:99 (v/v), two flow rates of the mobile phase (1.0 $ml \cdot min^{-1}$ and 0.5 $ml \cdot min^{-1}$) using various separation columns with a non-polar sorbent C18 operating at 30 °C. The goal was to obtain a symmetric peak of PAT, which should be well distinguished from HMF, the dominant interfering compound present in thermally processed apple products. The tested parameters are given in Tab. 1. Applying Column 1, two split peaks of PAT were obtained, presumably due to the silica sorbent without end-capping, which may be a source of secondary interactions between PAT and sorbent particles. Despite unique features of monolithic end-capped silica with a bimodal pore structure, Column 2 was not capable to sufficiently separate PAT and HMF that eluted together in one peak. Slight modifications of mobile phase composition and flow rate adjustment did not improve the separation of the two components on this column. Finally, Column 3 was selected, which allowed very good separation of the two peaks and much better peak symmetry for PAT. Isocratic elution with mobile phase of ACN-water in ratio 5:95 (v/v) at a flow rate of 1.0 $ml \cdot min^{-1}$ was used for the first 15 min of elution to reliably sepa-

Tab. 1. Variables determined for chromatographic resolution of patulin and hydroxymethylfurfural in a non-matrix model solution.

	Mobile phase composition (v/v) [%]	Mobile phase flow rate [$ml \cdot min^{-1}$]	Retention time [min]	Peak symmetry of patulin
Column 1	ACN-water 5:95	1.0	PAT 10.218 + 11.135	–
	ACN-1% AA 1:99	1.0	PAT 14.590 + 15.519	–
Column 2	ACN-water 20:80	1.0	PAT 3.024	0.667
	ACN-water 5:95	1.0	PAT 3.041	0.671
	ACN-water 5:95	0.5	HMF + PAT 6.003	0.720
	ACN-water 1:99	0.5	HMF + PAT 7.245	0.731
	ACN-1% AA 1:99	0.5	HMF + PAT 9.111	0.754
Column 3	ACN-water 5:95	1.0	HMF 10.219, PAT 12.738	0.960
	ACN-1% AA 1:99	1.0	HMF 15.118, PAT 17.901	0.883

HMF – hydroxymethylfurfural, PAT – patulin, ACN – acetonitrile, AA – acetic acid.
Column 1: Zorbax SB-C18, 250 mm × 4.6 mm, 5 μm particle size (Agilent Technologies); Column 2: Chromolith Performance RP-18e, 100 mm × 4.6 mm (Merck); Column 3: Purospher STAR RP-18e, 250 mm × 4.6 mm, 5 μm particle size (Merck).

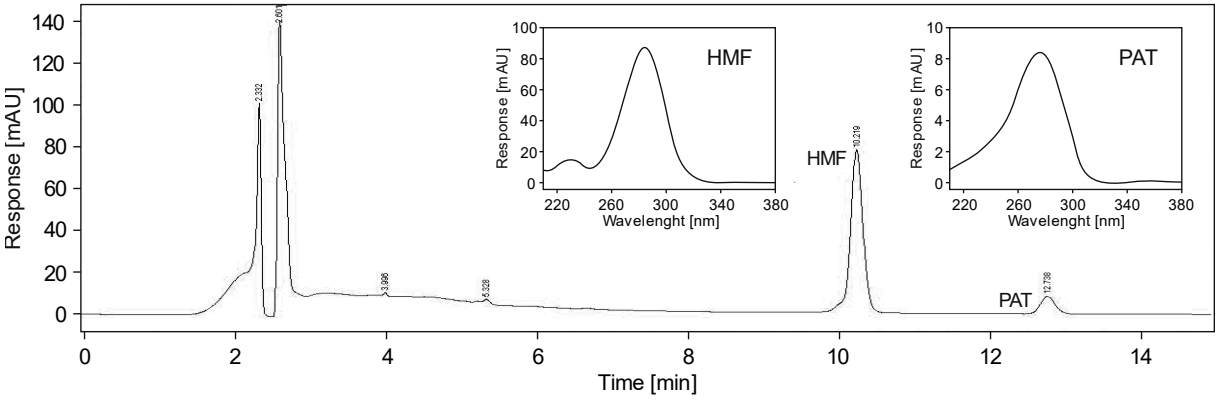

Fig. 1. Chromatogram and absorption spectra of hydroxymethylfurfural and patulin in a model solution.

HMF – hydroxymethylfurfural, PAT – patulin.

Tab. 2. Recovery rates of clean-up columns in patulin determination.

	SPE column	
	Oasis HLB (Waters)	EASIMIP Patulin (R-Biopharm Rhône)
Volume taken to SPE [ml]	2.50	5.00
Volume before injection [ml]	0.25	0.50
Theoretical level of patulin contamination	Recovery rate [%]	
10 $\mu g \cdot kg^{-1}$	89 ± 11 (n = 5)	83 ± 17 (n = 5)
50 $\mu g \cdot kg^{-1}$	79 ± 8 (n = 4)	90 ± 17 (n = 4)
100 $\mu g \cdot kg^{-1}$	92 ± 6 (n = 3)	91 ± 2 (n = 3)

SPE – solid phase extraction, n – number of measurements.

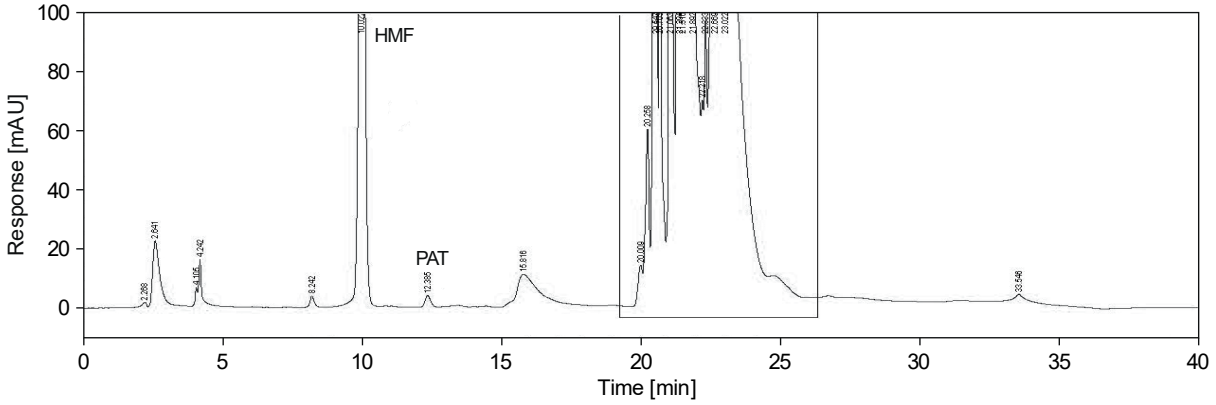

Fig. 2. Chromatogram of apple purée with a patulin content of 50 $\mu g \cdot kg^{-1}$.

Framed peaks represent extracted matrix components with higher retention than patulin. HMF – hydroxymethylfurfural, PAT – patulin.

rate HMF and PAT with an average retention time difference of 2.5 min (Fig. 1). Analysing purée samples, gradient elution with addition of ACN was involved after 15 min of elution, in order to elute from the column other more retentive compounds originating from the matrix.

Regarding OTA, an analytical procedure previously developed in our laboratory for cereals and cereal-based products [16] was used as described in the foregoing section „Final HPLC conditions ".

Optimization of extraction procedure

Patulin

AOAC Official Method 2000.02 [17] employing HPLC with UV detection is applicable to determine PAT at > 25 $ng \cdot g^{-1}$ in clear and cloudy apple juices and apple purée using two-stage liquid-liquid extraction (LLE) with ethyl acetate and washing with sodium carbonate solution for PAT in clear juices. Cloudy juice and purée are pretreated with pectinase to improve their clarity and increase recovery of PAT. Using this technique, various *LOD* values were attained in the range of 1.0–5.0 $\mu g \cdot l^{-1}$ for juices [18–20]. Less laborious approaches were also investigated, involving single-stage extraction of PAT from apple juices with solvents such as ethyl acetate, ACN, or a mixture of ACN and chloroform [21–23], with comparable *LOD* values. The newer version of the European standard EN 15890:2010 [24] offers LLE with ethyl acetate and hexane in presence of sodium carbonate and sodium sulfate, and subsequent purification of the fruit purée extract by SPE on silica gel. That method was validated for a range of products for babies including also products other than apple juices and purées.

In the present work, the widely used LLE procedure was excluded and SPE for clean-up was studied on the basis of IAC and MIP principle. First, several combinations of extraction solvents (ACN, ethyl acetate) with various treatment (orbital shaker/ultrasonic bath; centrifugation/filtration; vacuum/nitrogen evaporation) were tested, though yielding a coloured and pectin-like residue. Therefore, pectinase was used at 40 °C for 2 h for purée depectinization and liquification. After centrifugation and filtration, the obtained homogenous clear extract was submitted to clean-up on SPE Oasis HLB columns (the stationary phase

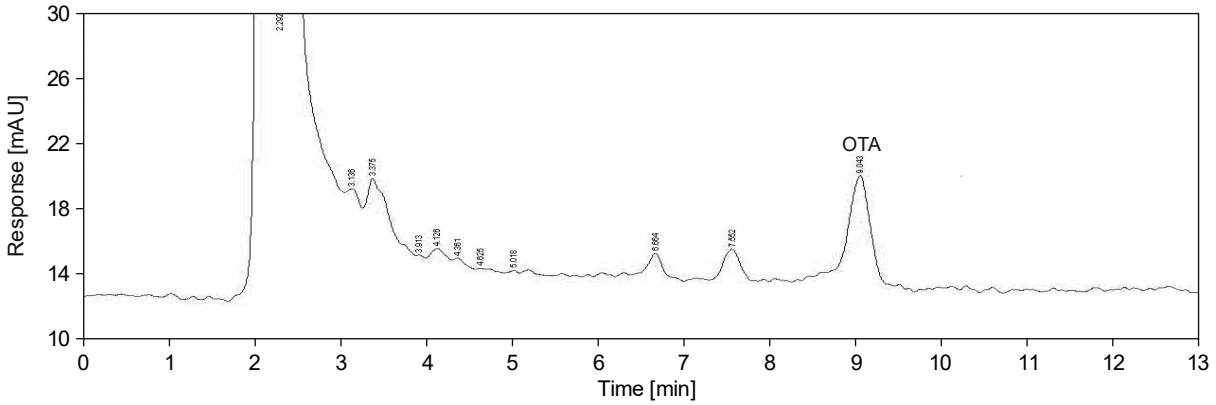

Fig. 3. Chromatogram of multicomponent purée with a natural ochratoxin A content of 0.12 $\mu g \cdot kg^{-1}$.
The purée consisted of apple, banana and buckwheat. OTA – ochratoxin A.

being a macro-porous co-polymer of lipophilic divynil benzene and hydrophilic N-vinyl pyrrolidone) and on EASIMIP Patulin columns. The columns were handled according to recommendations [15, 25]. An optimal volume of the extract taken for SPE was examined simultaneously with a volume necessary to dissolute the residue obtained after solvent evaporation. Both tested SPE columns showed good efficacy in retention capacity and recovery rate of PAT (Tab. 2). However, due to economy, Oasis HLB columns were chosen for further work.

Because of presence of some matrix constituents in the obtained extracts, the analytical run was adapted to avoid accumulation of more non-polar components together with PAT in the separation column. Therefore, after isocratic elution that took approximately 15 min, gradient elution was involved with pure ACN for next 15 min. As can be seen in Fig. 2, all undesired apple matrix components eluted between 20th and 25th min. After 30th min, the chromatographic system started adjusting the conditions for next analysis.

Ochratoxin A

The procedure for OTA extraction from fruit-cereal based purées, which is characterized in detail in the section „Preparation of sample for ochratoxin A analysis", closely resembles that the one that had been previously developed in our laboratory for cereals and cereal-based products [16]. In this work, only one small modification was done concerning blending. Because baby purée is a high-moisture matrix, slower mixing at ≈3.0 Hz instead of ≈3.7 Hz was used after addition of the extraction mixture. Extraction of OTA by SPE using specific IAC columns was utilized providing reliable results, as it was established in the previous work [16]. Fig. 3 illustrates the reliable separation of OTA from other matrix constituents by our method.

Performance of methods

The parameters for single laboratory validation of both methods and system suitability parameters are listed in Tab. 3. The calibration measurements were carried out with series of pure standard solutions of PAT (from 12.5 $ng \cdot ml^{-1}$ to 1 000 $ng \cdot ml^{-1}$) and OTA (from 0.1 $ng \cdot ml^{-1}$ to 62.5 $ng \cdot ml^{-1}$) using six content levels including blank sample. Individual calibration curves were constructed by linear least-squares regression with high linear relationship between the two variables fitting to a straight line (correlation coefficients $r^2 > 0.999$). The probability p-values of slope and intercept indicated significant non-zero statistics at $p \leq 0.05$ (95% confidence level) except for the value of PAT intercept (Tab. 3). The *LOD* and *LOQ* values were determined from five measurements of the blank matrix sample as defined in Eq. 1 and Eq. 2. The *LOD* value for PAT (5.8 $\mu g \cdot kg^{-1}$) was in a good agreement with other published methods, in which the same type of SPE column for clean-up was used (*LOD* ≈ 5–10 $\mu g \cdot l^{-1}$ for apple juices) [15, 26]. Repeatability (Tab. 3) was determined at two content levels of 10.5 $\mu g \cdot kg^{-1}$ and 47.0 $\mu g \cdot kg^{-1}$ for PAT, and 0.20 $\mu g \cdot kg^{-1}$ and 15.75 $\mu g \cdot kg^{-1}$ for OTA. The corresponding values of RSD_r complied with the official criteria for repeatability set for PAT ($RSD_r \leq 30$ % and $RSD_r \leq 15$ %, respectively), as well as for OTA ($RSD_r \leq 40$ % and $RSD_r \leq 20$ %, respectively) [27]. Recovery rates of both methods were determined using a purée sample spiked with the mycotoxin at three different content levels (Tab. 3) and they also fell within the interval of method performance requirements, i.e. 50–120 % for PAT

levels lower than 20 $\mu g \cdot kg^{-1}$ and 70–105 % for PAT levels within the interval of 20–50 $\mu g \cdot kg^{-1}$, as well as the recovery rate of 50–120 % for OTA levels lower than 1 $\mu g \cdot kg^{-1}$ and 70–110 % for OTA levels within the interval of 1–10 $\mu g \cdot kg^{-1}$ [27].

Based on validation characteristics, both methods provided good sensitivity and precision. The use of SPE offers complete clean-up of the purée extract with an expectation of lower material loss in comparison with methods using conventional LLE with clean-up and washing with a carbonate solution. However, the combination of LLE without carbonate followed by SPE on an Oasis HLB cartridge [28] could provide recovery rates as well as *LOD* and *LOQ* values comparable to those presented in this work.

Selectivity was studied in order to obtain optimal peak shape of the studied mycotoxins and differentiation from interfering matrix components during chromatographic separation. The study involved optimization of PAT and HMF separation for a sample without matrix and then for matrix-containing samples. Due to variability of multi-component purée samples, the numerical parameters of peak symmetry, peak resolution and selectivity factor are listed as an interval of acquired values (Tab. 3). As can be seen from the data in Tab. 3, the values of peak symmetry for OTA and PAT shifted towards 1, so almost every peak showed some degree of fronting or tailing. Excellent peak resolution of $R_s \geq 2.0$ was achieved for both mycotoxins at samples with high variability of composition. Similarly, the selectivity factors greater than 1 could demonstrate good separation power in the used system of adsorbent-mycotoxin-elution mixture, which was more stable for PAT. Identity and peak spectrum purity at PAT analysis was checked by DAD in each purée sample to confirm absence of an undesirable matrix component.

PAT control solution of 0.25 $\mu g \cdot ml^{-1}$ was used for estimation of peak area stability within two years by repetitive measurements each 3 months. Within this period, the conditions were influenced by new lots of PAT standard, solvents for mobile phase, SPE columns, as well as by changes in ambient temperature in course of seasons. The alterations also included a fluctuation of the analytical system. The results of observations were

Tab. 3. Validation and system suitability parameters for determination of ochratoxin A and patulin in fruit purée.

	Ochratoxin A	Patulin
Linear range [$\mu g \cdot kg^{-1}$]	0.12–15.75	6.9–170.0
Correlation coefficient r^2	0.9997	0.9999
Slope	0.96×10^{-5}	1.9345
Intercept	-38.48×10^{-5}	–4.6607
Standard error of slope	21.26×10^{-9}	0.021456
Standard error of intercept	0.41×10^{-4}	2.641905
p-value of slope	4.48×10^{-6}	0.91×10^{-7}
p-value of intercept	1.67×10^{-3}	0.15248
Limit of detection [$\mu g \cdot kg^{-1}$]	0.06	5.8
Limit of quantification [$\mu g \cdot kg^{-1}$]	0.12	6.9
Repeatability *RSDr* at different content level [%]	21.1 (0.20 $\mu g \cdot kg^{-1}$)	1.6 (10.50 $\mu g \cdot kg^{-1}$)
	5.3 (15.75 $\mu g \cdot kg^{-1}$)	0.1 (47.0 $\mu g \cdot kg^{-1}$)
Recovery rate at different content level [%]	78 ± 10 (0.60 $\mu g \cdot kg^{-1}$)	89 ± 11 (10.0 $\mu g \cdot kg^{-1}$)
	97 ± 8 (5.20 $\mu g \cdot kg^{-1}$)	79 ± 8 (50.0 $\mu g \cdot kg^{-1}$)
	105 ± 5 (10.20 $\mu g \cdot kg^{-1}$)	92 ± 6 (100.0 $\mu g \cdot kg^{-1}$)
Uncertainty *U* at different content level [%]	82 (0.20 $\mu g \cdot kg^{-1}$)	52 (10.5 $\mu g \cdot kg^{-1}$)
	18 (15.75 $\mu g \cdot kg^{-1}$)	9 (47.0 $\mu g \cdot kg^{-1}$)
Retention time* [min]	9.011 ± 0.066	12.403 ± 0.166
Peak width $W_{50.0}$* [min]	0.223–0.302	0.202–0.211
Efficiency (plates)*	7944–9251	18615–20656
Symmetry *	0.86–1.29	0.81–0.88
Resolution R_S*	5.84–6.77	6.90–7.93
Selectivity*	1.11–1.95	1.23–1.25

* – parameters acquired from data-processing software ChemStation (Agilent Technologies).
p-value of slope or intercept at 95% confidence level; peak width $W_{50.0}$ means the peak width at 50 % of the peak height.

Tab. 4. Content of mycotoxins in single- and multi-component purée samples.

Mycotoxin	Number of analysed samples	Number of positive samples	Content in positive samples [$\mu g \cdot kg^{-1}$]	EU limits for baby foods [$\mu g \cdot kg^{-1}$]
Ochratoxin A	25	2	0.12–0.20	0.5
Patulin	89	5	6.9–15.4	25 / 10 *

* – the level of 25 $\mu g \cdot kg^{-1}$ is assigned to apple purée intended for direct consumption; the level of 10 $\mu g \cdot kg^{-1}$ is assigned to apple purée for infants and young children and labelled and sold as such.

evaluated as relative standard deviation of ruggedness (*RSD*), which was 20.3 %. Stability of OTA control solution of 0.013 $\mu g \cdot ml^{-1}$ was observed during two months when a new lot of OTA standard and a new separation column were used. For these variations, the *RSD* value was 2.3 %. The small deviations of parameters acquired within these time intervals indicated good equipment integrity and stability of both methods in their routine usage.

Applicability of methods

Applicability of the suggested methods was verified by analysing a broad range of domestic purée products for babies during six years that comprised variability of the products. The results in Tab. 4 show that majority of samples contained no detectable amounts of mycotoxins. The positive findings of PAT (5.6 % incidence) were mostly related to intermediary products of apple purée, which contained a maximum of 15.4 $\mu g \cdot kg^{-1}$ PAT. Four out of five positive samples contained PAT at a level of *LOQ* (approximately 6.9 $\mu g \cdot kg^{-1}$). Regarding OTA, the highest level of 0.20 $\mu g \cdot kg^{-1}$ was recorded in one complex purée sample made from apple, banana, mango and cereals. The sample with the second highest OTA content (0.12 $\mu g \cdot kg^{-1}$) was a purée made from apple, banana and buckwheat. Incidence of OTA in the bulk of analysed samples was 8.0 %. None of the examined samples did show any co-occurrence of both mycotoxins.

Concerning other similar studies to compare, 21 fruit purée samples for babies were tested in Tunisia with no detectable PAT contamination [29]. A later Tunisian study revealed 28 % incidence of PAT contamination in baby foods, in particular in juices, in which the concentration of PAT exceeded the tolerable limit of 10 $\mu g \cdot l^{-1}$ [30]. In a Portuguese report that included apple-based food products for babies from conventional and organic sources, one sample containing PAT at a level slightly below the EU limit (9.1 $\mu g \cdot kg^{-1}$) was found [31]. Barreira et al. [32] found PAT contamination in 23 % of tested apple purée samples, at contents in the range of 1.2–42.0 $\mu g \cdot kg^{-1}$. Furthermore, ten apple purée samples labelled as organic food, which were inspected in Italy, were found to contain PAT at levels 17.7 $\mu g \cdot kg^{-1}$ and 13.1 $\mu g \cdot kg^{-1}$ [33]. By contrast, alarming contamination with PAT was found in apple purée samples in Argentina, at 50% incidence and with average content of 123.0 $\mu g \cdot kg^{-1}$ [34].

CONCLUSION

The methods developed and validated in this study enrich the armamentarium of analytical methods for quantification of PAT and OTA in fruit purées with or without addition of cereals. Parameters of the methods are fully comparable with the currently most used procedures. The advantages of our methods are: (1) simplified sample preparation without LLE, which implies faster manipulation without possible material loss; (2) more complete clean-up of extracts by SPE; (3) improved control of peak purity by DAD, which is important in particular at PAT analysis of multi-component fruit-based samples.

Both methods were optimized and validated independently, and were utilized in a long-term monitoring programme of commercial fruit-based and fruit-cereal purées for babies produced in Slovakia. In frames of this, low incidence of PAT and OTA (5.6 % and 8.0 %, respectively) was found except for one sample of apple purée (intermediary product) containing 15.4 $\mu g\ kg^{-1}$ PAT, thus exceeding the EU limit value of 10 $\mu g \cdot kg^{-1}$ established for PAT content in baby products. Co-occurrence of both mycotoxins was not confirmed in any analysed sample with fruit-cereal components. This work contributes to knowledge of occurrence of PAT and OTA in fruit-based and fruit-cereal purées for babies.

Acknowledgement

This publication was supported by the Operational program Integrated Infrastructure within the project: Demand-driven research for the sustainable and innovative foods, Drive4SIFood, 313011V336, cofinanced by the European Regional Development Fund.

REFERENCES

1. Drush, S. – Ragab, W.: Mycotoxins in fruits, fruit juices and dried fruits. Journal of Food Protection, *66*, 2003, pp. 1514–1527. DOI: 10.4315/0362-028x-66.8.1514.
2. Moss, M. O.: Fungi, quality and safety issues in fresh fruits and vegetables. Journal of Applied Microbiology, *104*, 2008, pp. 1239–1243. DOI: 10.1111/j.1365-2672.2007.03705.x.
3. Barkai-Golan, R. – Paster, N. (Ed.): Mycotoxins in fruits and vegetables. San Diego : Academic Press, 2008. ISBN: 978-0-12-374126-4. DOI: 10.1016/B978-0-12-374126-4.X0001-0.
4. Moake, M. M. – Padilla-Zakour, O. I. – Worobo, R. W.: Comprehensive review of patulin control methods in foods. Comprehensive Reviews in Food Science and Food Safety, *4*, 2005, pp. 8–21. DOI: 10.1111/j.1541-4337.2005.tb00068.x.
5. Senyuva, H. Z. – Gilbert, J.: Identification of fumonisin B_2, HT-2 toxin, patulin and zearalenone in dried figs by liquid chromatography-time-of-flight mass spectrometry and liquid chromatography-mass spectrometry. Journal of Food Protection, *71*, 2008, pp. 1500–1504. DOI: 10.4315/0362-028x-71.7.1500.
6. Reddy, K. R. – Spardaro, D. – Lore, A. – Gullino, M. L. – Garibaldi, A.: Potential of patulin production by *Penicillium expansum* strains on various fruits. Mycotoxin Research, *26*, 2010, pp. 257–265. DOI: 10.1007/s12550-010-0064-5.
7. Acar, J. – Gökmen, V. – Taydas, E. E.: The effects of processing technology on the patulin content of juice during commercial apple juice concentrate production. Zeitschrift für Lebensmittel Untersuchung und Forschung A, *207*, 1998, pp. 328–331. DOI: 10.1007/s002170050341.
8. Lerda, D.: Mycotoxin factsheet. 4th edition. JRC Technical Notes. Geel : Joint Research Centre, 2011. <https://ec.europa.eu/jrc/sites/jrcsh/files/Factsheet%20Mycotoxins_2.pdf>
9. Ochratoxin A. In: Safety evaluation of certain mycotoxins in food. FAO Food and Nutrition Paper 74, WHO Food Additives Series 47. Geneva : World Health Organisation, 2001, pp. 281–415. ISBN: 9241660473, 9251046646. <http://www.fao.org/3/a-bc528e.pdf>
10. Al-Hazmi, N. A.: Determination of patulin and ochratoxin A using HPLC in apple juice samples in Saudi Arabia. Saudi Journal of Biological Sciences, *17*, 2010, pp. 353–359. DOI: 10.1016/j.sjbs.2010.06.006.
11. Joosten, H. M. L. J. – Goetz, J. – Pittet, A. – Schellenberg, M. – Bucheli, P.: Production of ochratoxin A by *Aspergillus carbonarius* on coffee cherries. International Journal of Food Microbiology, *65,* 2001, pp. 39–44. DOI: 10.1016/S0168-1605(00)00506-7.
12. Commission Recommendation of 11 August 2003 on the prevention and reduction of patulin contamination in apple juice and apple juice ingredients in other beverages (notified under document number C(2003) 2866). Official Journal of the European Union, *46*, 2003, L203, pp. 54–59. ISSN: 1725-2555. <http://data.europa.eu/eli/reco/2003/598/oj>
13. Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. Official Journal of the European Union, *49*, 2006, L364, pp. 5–24. ISSN: 1725-2555. <http://data.europa.eu/eli/reg/2006/1881/oj>
14. MacDonald, S. – Long, M. – Gilbert, J. – Felgueiras, I.: Liquid chromatographic method for determination of patulin in clear and cloudy apple juices and apple puree: collaborative study. Journal of AOAC International, *83*, 2000, pp. 1387–1394. DOI: 10.1093/jaoac/83.6.1387.
15. Eisele, T. A. – Gibson, M. Z.: Syringe-cartridge solid-phase extraction method for patulin in apple juice. Journal of AOAC International, *86*, 2003, pp. 1160–1163. DOI: 10.1093/jaoac/86.6.1160.
16. Belajová, E. – Rauová, D.: Single laboratory-validated HPLC methods for determination of ochratoxin A, fumonisin B1 and B2, zearalenone and deoxynivalenol in cereals and cereal-based foods. Journal of Food and Nutrition Research, *49*, 2010, pp. 57–68. ISSN: 1336-8672. <https://www.vup.sk/download.php?bulID=123>
17. AOAC No. 49.7.03 Official Method 2000.02. Patulin in clear and cloudy apple juice and apple puree. Liquid Chromatographic Method, First Action 2000. Journal of AOAC International, *83*, 2000, pp. 1387. ISSN: 1060-3271.
18. Rosinska, D. – Vierikova, M. – Lehotay, J.: Determination of patulin in apple products using HPLC with photodiode array detector and ultra performance liquid chromatography with electrospray-tandem mass spectrometry. Journal of Liquid Chromatography and Related Technologies, *32*, 2009, pp. 500–511. DOI: 10.1080/10826070802671358.
19. Gökmen, V. – Acar, J.: Simultaneous determination of 5-hydroxymethylfurfural and patulin in apple juice by reversed-phase liquid chromatography. Journal of Chromatography A, *847*, 1999, pp. 69–74. DOI: 10.1016/s0021-9673(99)00133-8.
20. Versari, A. – Parpinello, G. P. – Mattioli, A. U.: Survey in patulin contamination in Italian apple juice from organic and conventional agriculture. Journal of Food Technology, *5*, 2007, pp. 143–146. ISSN: 1684-8462. <http://medwelljournals.com/abstract/?doi=jftech.2007.143.146>.
21. Sargenti, S. R. – Almeida, C. A. A.: Determination of patulin in apple juice by HPLC using a simple and fast sample preparation method. Eclética Química, *35*, 2010, pp. 14–21. DOI: 10.1590/S0100-46702010000200002.
22. González-Osnaya, L. – Soriano, J. M. – Moltó, J. C. – Manes, J.: Exposure to patulin from consumption of apple-based products. Food Additives and Contaminants, *24*, 2007, pp. 1268–1274. DOI: 10.1080/02652030701361556.
23. Farhadi, K. – Maleki, R.: Dispersive liquid-liquid microextraction followed by HPLC-DAD as an efficient and sensitive technique for the determination of patulin from apple juice and concentrate samples. Journal of Chinese Chemical Society, *58*, 2011,

pp. 340–345. DOI: 10.1002/jccs.201190035.
24. EN 15890:2010. Foodstuffs – Determination of patulin in fruit juice and fruit based purée for infants and young children – HPLC method with liquid/liquid partition cleanup and solid phase extraction and UV detection. Brussels : CEN European Committee for Standardization, 2010.
25. EASIMIP™ Patulin, Product Code: P250/P250B. Molecularly imprinted polymer columns for use in conjuction with HPLC. For *in vitro* use only. Glasgow : R-Biopharm Rhône, sine anno. <https://food.r-biopharm.com/wp-content/uploads/sites/2/2012/06/p250_easimip-patulin-ifu-v5_2018-09.pdf>
26. Trucksess, M. W. – Tang, Y.: Solid-phase extraction method for patulin in apple juice and unfiltered apple juice. Journal of AOAC International, *82*, 1999, pp. 1109–1113. <https://doi.org/10.1093/jaoac/82.5.1109>
27. Commission Regulation (EC) No 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. Official Journal of the European Union, *49*, 2006, L70, pp. 12–34. ISSN: 1725-2555. <http://data.europa.eu/eli/reg/2006/401/oj>
28. De Clercq, N. – Van Pamel, E. – Van Coillie, E. – Vlaemynck, G. – Devlieghere, F. – De Meulenaer, B. – Daeseleire, E.: Optimization and validation of a method without alkaline clean-up for patulin analysis on apple puree agar medium (APAM) and apple products. Food Analytical Methods, *9*, 2016, pp. 370–377. DOI: 10.1007/s12161-015-0190-y.
29. Mhadhbi, H. – Bouzouita, N. – Martel, A. – Zarrouk, H.: Occurence of mycotoxin patulin in apple-based products marketed in Tunisia. Journal of Food Protection, *70*, 2007, pp. 2642–2645. DOI: 10.4315/0362-028x-70.11.2642.
30. Zaied, C. – Abid, S. – Hlel, W. – Bacha, H.: Occurence of patulin in apple-based-foods largely consumed in Tunisia. Food Control, *31*, 2013, pp. 263–267. DOI: 10.1016/j.foodcont.2012.10.005.
31. Cunha, S. C. – Faria, M. A. – Fernandes, J. O.: Determination of patulin in apple and quince products by GC-MS using $^{13}C_{5-7}$ patulin as internal standard. Food Chemistry, *115*, 2009, pp. 352–359. DOI: 10.1016/j.foodchem.2008.11.074.
32. Barreira, M. J. – Alvito, P. C. – Almeida, C. M. M.: Occurrence of patulin in apple-based-foods in Portugal. Food Chemistry, *121*, 2010, pp. 653–658. DOI: 10.1016/j.foodchem.2009.12.085.
33. Ritieni, A.: Patulin in Italian commercial apple products. Journal of Agricultural and Food Chemistry, *51*, 2003, pp. 6086–6090. DOI: 10.1021/jf034523c.
34. Funes, G. J. – Resnik, S. L.: Determination of patulin in solid and semisolid apple and pear products marketed in Argentina. Food Control, *20*, 2009, pp. 277–280. DOI: 10.1016/j.foodcont.2008.05.010.

Received 11 May 2020; 1st revised 20 July 2020; 2nd revised 6 October 2020; accepted 26 October 2020; published online 4 November 2020.