

# Assessment of Patulin Content in Apple Puree and Apple and Fruit Puree by High Performance Liquid Chromatography

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**Abstract:** An analytical method was developed and validated for determination of patulin in apple puree by HPLC. Extraction and clean-up of patulin from clear extract are achieved on AFFINIMIP<sup>®</sup> SPEPATULIN cartridges. Patulin is then separated on a Hypersil GOLD column 150 mm × 4 mm, 5 μm and detected at 276 nm. The recovery in the range of 5 μg/kg-80 μg/kg was 81.47%. The limit of detection (LOD) was 1.36 μg/kg, and the limit of quantification (LOQ) was 4.55 μg/kg. The patulin content of the commercial samples of apple puree and samples of apple and fruit puree for infants and young children as well as the samples of apple puree prepared from two apple varieties intended for processing (Jonathan, Florina) and obtained from conventional and uncertified organic cultures has been evaluated in this paper. The 44.83% patulin concentration of the analyzed samples were under the maximum level of the European Commission Regulation (EC) 1881/2006, in 46.55% of the analyzed samples patulin was not detected and in 8.62% of samples patulin concentration was lower than LOQ (European Commission, 2006a) Patulin was not detected in samples of apple puree intended for infants and young children consumption.

**Key words:** Patulin, HPLC-DAD, apple puree, method performance parameters.

## 1. Introduction

Mycotoxins are metabolites of fungi, which are able to cause acute or chronic toxic effects (carcinogens, mutagens, teratogens) on animals and humans. Patulin is a mycotoxin produced by a range of fungal species, generally *Penicillium*, *Aspergillus* and *Byssoschlamys*, of which *Penicillium expansum* is probably the most prevalent species. Patulin was discovered as contaminant in fruits and vegetables, but moldy apple and their processed products are the main contamination source of this mycotoxin. Initially, patulin was considered as having therapeutic effect, as a result of antibiotic

properties. But in 1960s it was reclassified as mycotoxin because of its toxicity [1]. The investigations undertaken have shown that patulin has mutagenic, neurotoxic, immunotoxic, genotoxic effects on rodents and different effects on gastrointestinal tract, such as distension, ulceration and bleeding [2]. International Agency for Research on Cancer (IARC-Geneva, Switzerland) has classified patulin as group 3 (Not classifiable as to its carcinogenicity to humans) concerning its potential as a human carcinogen [3].

The Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives (JEFCA) proposed a provisional maximum tolerable daily intake (PMDTI) of 0.4 μg/kg body weight/day, based on reproductive and carcinogenicity studies and its toxicity [4].

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Patulin appears mainly in moldy fruits. Although the presence of mold does not necessarily imply the presence of patulin in fruit, it indicates the possibility. In some circumstances, the inside development of molds may be due to insects or other invasions of healthy tissues, leading to the appearance of patulin in fruit, which externally appears to be unaffected. However, patulin may occur in hit fruits after the storage under controlled atmosphere and exposure to the environmental conditions, with or without fruit pulp alteration. Washing fruit or removing of moldy tissue immediately before pressing will not remove patulin in the fruit, because it diffuses in the apparently healthy tissue [5].

According to IUPAC, patulin is chemically known as 4-hydroxy-4H-furo[3,2-c]pyran-2(6H)-one and it is an unsaturated heterocyclic lactone with molecular weighting 154, stable in acidic medium but unstable in alkaline medium. Patulin is a colorless compound, with a melting point of 110 °C. Maximum absorbance in UV is at 276 nm [6, 7].

Patulin is very soluble in water and in most organic solvents. It is stable in diluted acids and resistant to temperatures up to 125 °C, in the pH range of 3.5-5.5 [8].

The presence of patulin in 144 apple-based-foods in Portugal was studied by Barreira [9]. Patulin was detected in 32 samples, its concentration being in the range of 1.2-42 µg/kg. In the mentioned study patulin was not detected in infant drinks, but was quantified in five homogenised apple puree intended to feed infants and young children. At the same time, 67% positive samples were detected in cloudy juices and 13% in clear juices.

Presence of patulin in organic and conventional apple-based food marketed in Catalonia, as well as the exposure of infants and young children, were studied by Piqué [10]. The estimated daily intake of patulin for infants and young children (0-3 years), children (4-18 years) and adults (19-66 years), was under the provisional maximum tolerable daily intake (PMTDI)

of 0.4 µg/kg by weight.

This paper describes the development and validation of a method for determination of patulin by HPLC-DAD from apple puree and apple and fruit puree, purchased from supermarkets and pharmacies in Bucharest. At the same time, apple puree samples are prepared within the Pilot Experiments Plant for Vegetables and Fruits Processing from INCDBA-IBA Bucharest, using two apple varieties intended for processing, from conventional and uncertified organic cultures.

## **2. Materials and Methods**

### *2.1 Food Matrices and Samples*

Samples used in this study were purchased from the market and pharmacies shelves in Bucharest as well as apple puree samples which have been prepared within the Pilot Experiments Plant for Vegetables and Fruits Processing from INCDBA-IBA Bucharest. In these experiments, two apple varieties intended for processing (*Jonathan*, *Florina*) and obtained by conventional and uncertified organic cultures have been used. The apples have been purchased from the market of local farmers. Technological flowchart of apple puree processing has the following technological operations: washing, cutting, blanching, pulping, dosing (in glass recipients capacity 220 mL), closing, pasteurization (10 minutes at 100 °C), cooling (at 40 °C) and storage.

### *2.2 Methods*

Samples of apple puree and apple and fruit puree were chemically-physically analysed using the following methods:

SR ISO 2173: 2008. Fruit and vegetable products. Determination of soluble dry matter by refractometry;

SR ISO 1842:2008. Fruit and vegetable products. Determination of pH.

### *2.3 Determination of Patulin*

#### *2.3.1 Reagents and Materials*

HPLC grade glacial acetic acid, HPLC grade ethyl

acetate and HPLC grade methanol have been purchased from SIGMA-ALDRICH. Optigrade acetonitrile have been purchased from LGC Standards and ultrapure water was obtained in house using ELGA water ultrapurification system. For calibration curves, patulin standard (5 mg, purity = 99.5%) was obtained from Sigma-Aldrich (St Louis, MO. USA).

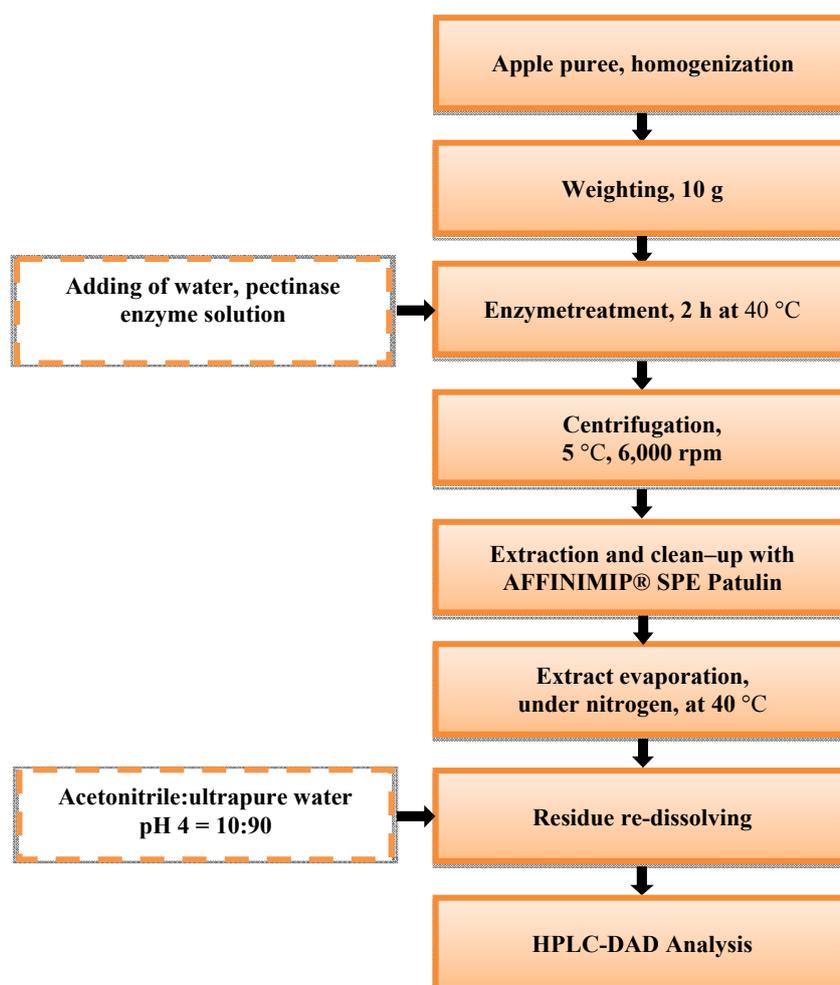
AFFINIMIP® SPE Patulin cartridges (6 mL-200 mg) and pectinase enzyme have been obtained from AffiniseP-Polyintell (Val-de-Reuil, France).

### 2.3.2 Sample Preparation

The steps of the method of determination of patulin in apple puree are shown as below, including sample preparation, weighting, enzyme treatment, centrifugation, extraction and clean-up on AFFINIMIP® SPE Patulin cartridges, extract

evaporation, residue redissolving, and HPLC-DAD analysis (Fig. 1).

Sample preparation is based on Application note-AFFINIMIP® SPE *Patulin* 6 mg/200 mg-apple puree [11]. In a 50 mL centrifuge vial, 10 g of apple puree sample, 150 µL of pectinase enzyme solution and 10 mL ultrapure water were mixed. Sample is maintained in a water bath at 40 °C for 2 hours, and then centrifuged at 6,000 rpm, at 5 °C for 25 min. The supernatant (5 mL) is purified on AFFINIMIP® SPE Patulin cartridge, which has been pre-conditioned with 2 mL acetonitrile and 1 mL of ultrapure water. In order to remove interferences, it need to be passed through the cartridge of 4 mL water -0.1% acetic acid, 4 mL of ultrapure water, then apply vacuum about 10 s. Further, 500 µL ethyl ether are passed through the



**Fig. 1** Flowchart with steps of the method of determination of patulin in apple puree by HPLC-DAD.

cartridge and finally patulin was eluted with 2 mL acetonitrile containing 0.1% acetic acid. The elution fraction is evaporated near dryness under a nitrogen atmosphere at 40 °C, and then re-dissolved in acetonitrile:ultrapure water (pH = 4) = 10:90v/v.

### 2.3.3 Parameters and Conditions of HPLC-DAD Method for Determination of Patulin in Apple Puree

A *Surveyor Plus (Thermo Finnigan)* high performance liquid chromatograph was used (vacuum degasser, quaternary pump, autosampler with PELTIER sample temperature control, column compartment with PELTIER temperature control, diode array detector, Chrom Quest 4.2 software for data acquisition and data processing). The separation was performed at 25 °C, on a C18 (Hypersil GOLD 150 mm × 4 mm, 5 μm) with a Hypersil Gold guard column (10 mm × 4 mm, 5 μm). The composition of mobile phase was water acetonitrile (95:15, v/v). The injection volume was 25 μL; the flow rate of the mobile phase was 1.0 mL/min. And the detection wavelength was 276 nm. Peak identification was based on retention time, spectral information and spiking technique. Peak quantification was based on the external standard method, using calibration curve.

## 3. Results and Discussions

The developed method was validated according to

the provisions of the Regulation (EC) No 401/2006 [12].

### 3.1 Linearity

The linearity of the method for determination of patulin in apple puree was evaluated by the method of least squares (linear regression method), for calibration with external standard. For calibration curve, patulin standard (5 mg) was dissolved in ethyl acetate and then this solution was diluted with ethanol. Patulin concentration in this solution (10 μg/mL) was tested on the basis of maximum absorbance spectrum between 250 nm-350 nm (Fig. 2), based on Eq. (1):

$$\rho_{pat} = A_{max}x \frac{Mx100}{\epsilon x \delta} \quad (1)$$

where:

$\rho_{pat}$ —concentration of patulin solution, expressed as μg/mL;

$A_{max}$ —corresponding absorbance of maximum absorption curve (276 nm);

$M$ —patulin molecular weight ( $M = 154.12$  g/mol);

$\epsilon$ —relative molar absorption coefficient of patulin in ethanol (in this case 1,460 m<sup>2</sup>/mol, according to AOAC Official Methods, 1995, Natural Toxins, Patulin, 49.6.01. C (d))

$\delta$ —the optical path length of quartz cuvette in cm.

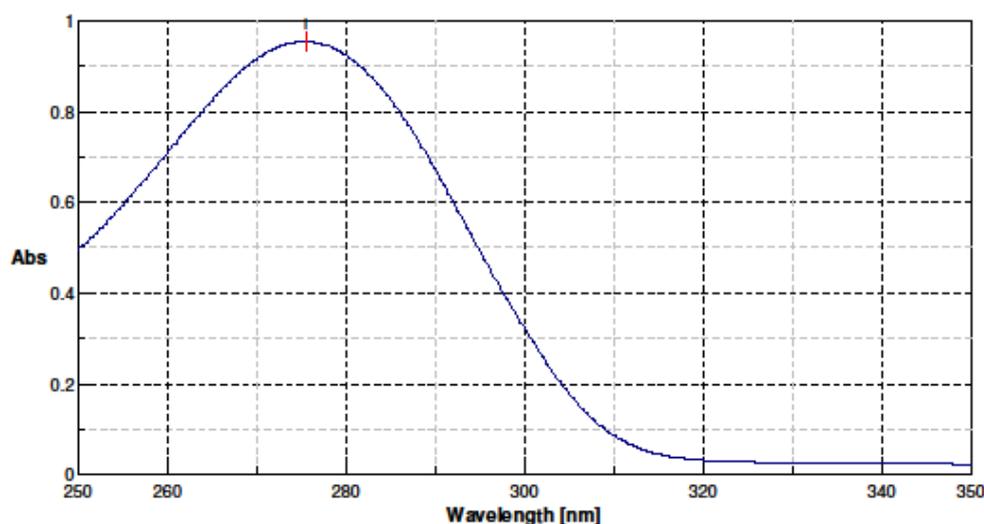


Fig. 2 Spectrum of patulin alcoholic solution.

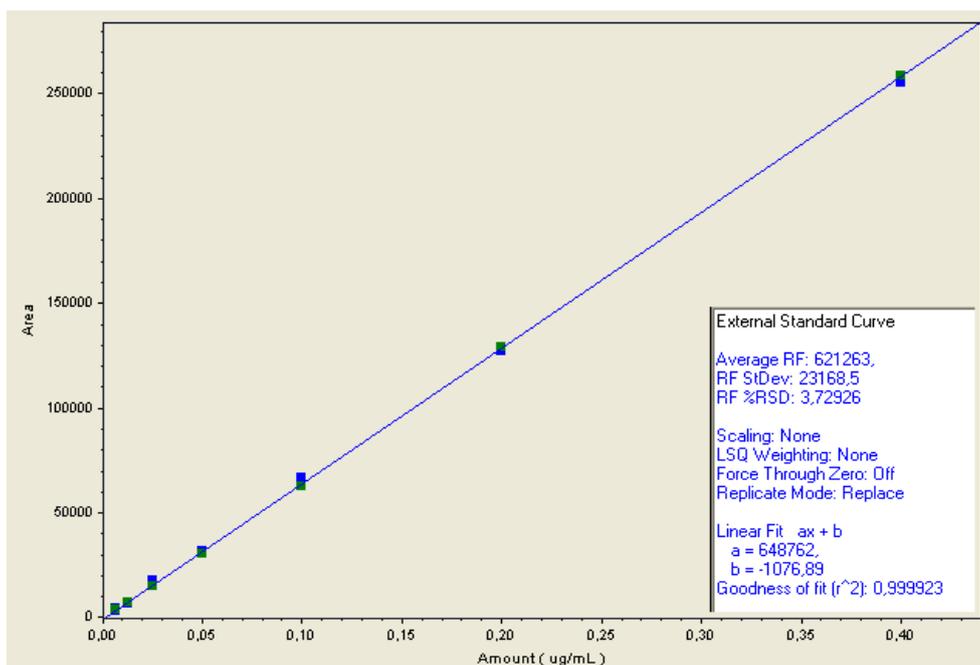


Fig. 3 Patulin calibration curve.

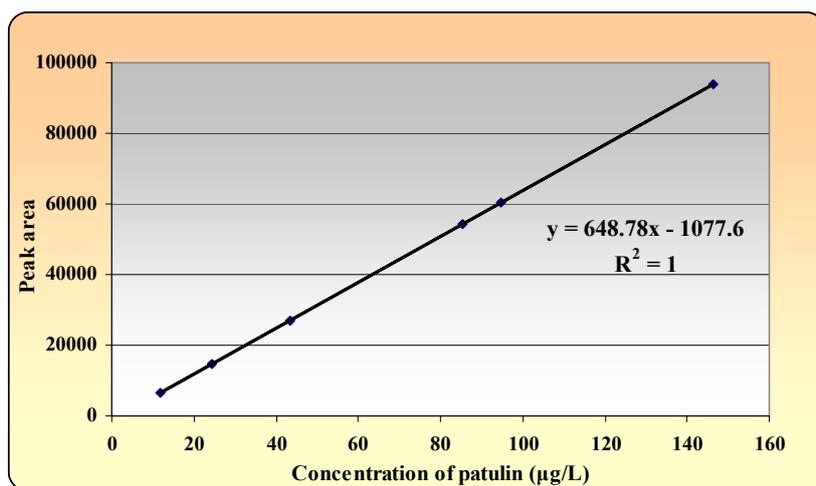


Fig. 4 Linearity domain of method for patulin determination in apple puree, by high performance liquid chromatography.

In the case of the achieved solution  $A_{max} = 0.9479$ , the concentration of solution was  $\rho_{pat} = 10.00 \mu\text{g/mL}$ .

Seven patulin standard levels (with three replicate injections from each level), in concentration range from  $6.25 \mu\text{g/L}$  to  $400 \mu\text{g/L}$ , have been used for the calibration curve (Fig.3) [13].

The following linear regression values were obtained from the linear regression equation:  $y = 648762x - 1076.89$ ; regression coefficient  $R^2 = 0.999923$ , slope  $b = -1076.89$ , intercept  $a = 648762$ .

Linearity range of the method was determined by

analysis of some blank samples of apple puree, which have been spiked with the following patulin concentrations:  $5 \mu\text{g/kg}$ ,  $10 \mu\text{g/kg}$ ,  $20 \mu\text{g/kg}$ ,  $40 \mu\text{g/kg}$ ,  $50 \mu\text{g/kg}$  and  $80 \mu\text{g/kg}$ . By plotting patulin concentration in the injected test sample versus the peak area, the following linear regression equation was obtained  $y = 648.78x - 1077.6$ , with regression coefficient  $R^2 = 1$  (Fig. 4).

The linearity domain of method for patulin determination from apple puree, by high performance chromatography is  $11.69 \mu\text{g/L}$ - $146.42 \mu\text{g/L}$ , which is

equivalent to the range of 5 µg/kg-80 µg/kg of patulin in apple puree.

### 3.2 Recovery

Apple puree samples in which patulin which was not detected (blank samples), were spiked with patulin (using patulin standard solution for calibration, with patulin concentration of 1 µg/mL) in the following concentrations: 5 µg/kg, 10 µg/kg, 20 µg/kg, 40 µg/kg, 50 µg/kg, 60 µg/kg and 80 µg/kg. These samples were analysed to determine patulin concentration (each concentration level was analyzed in 6 parallel prepared samples), and the following mean *recovery* values were obtained:

- 91.36%, in the case of patulin concentrations, < 20 µg/kg;
- 81.60%, in the range of patulin concentrations, 20-50 µg/kg;
- 75.17%, in the case of patulin concentrations, > 50 µg/kg.

In the concentration range of 5 µg/kg and 80 µg/kg, *mean recovery* was 81.47%. According to the obtained results, *mean recovery*, for the ranges of concentrations mentioned above, was under the provisions of the Regulation (EC) No. 401/2006 [12].

### 3.3 Precision, Repeatability and i-Intra-laboratory Reproducibility

To evaluate the instrument precision, 6 consecutive injections of apple puree samples (blank) spiked with

patulin, at two concentration levels of 5 µg/kg and 10 µg/kg, have been used. The following parameters have been calculated: patulin mean concentration, mean recovery, standard deviation in repeatability conditions SD(r) and relative standard deviation in repeatability conditions RSD(r) (Table 1).

In order to verify method precision, 6 parallel samples of apple puree (blank) spiked with patulin at two concentration levels of 10 µg/kg and 40 µg/kg were analysed by the same analyst, in the same laboratory and using the same instrument. The following parameters have been calculated: patulin mean concentration, standard deviation in repeatability conditions SD(r), relative standard deviation in repeatability conditions RSD(r), repeatability limit and expanded uncertainty (Table 2).

For evaluating the intra-laboratory reproducibility, 6 samples have been analyzed by two analysts (analyst A—3 samples, analyst B—3 samples) of apple puree (blank) spiked with patulin, at one concentration level of 20 µg/kg performed in the same laboratory and using the same instrument. The following parameters have been calculated: patulin mean concentration, standard deviation in reproducibility conditions SD(R), relative standard deviation in reproducibility conditions RSD(R), reproducibility limit and expanded uncertainty (Table 3).

According to the obtained results, relative standard deviation achieved under repeatability conditions RSD(r) and relative standard deviation achieved under

**Table 1 Statistical parameters for repeatability (instrument precision), in the case of method for determination of patulin in apple puree.**

Sample name	Spiking level (µg/kg)	Mean value C <sub>Patulin</sub> (µg/kg)	Mean value recovery (%)	SD(r) (µg/kg)	RSD(r) (%)
Apple puree	5 (n = 6)	4.67	93.14	0.12	2.66
Apple puree	10 (n = 6)	9.03	90.27	0.08	0.86

**Table 2 Statistical parameters for repeatability (method precision), in the case of method for determination of patulin in apple puree.**

Sample name	Spiking level (µg/kg)	Mean value C <sub>Patulin</sub> (µg/kg)	SD(r) (µg/kg)	RSD(r) (%)	Repeatability limit, r (µg/kg)	Expanded uncertainty (µg/kg)	Confidence interval (µg/kg)
Apple puree	10 (n = 6)	10.44	0.67	6.40	1.87	1.36	10.44 ± 1.36
Apple puree	40 (n = 6)	40.69	0.97	2.39	2.73	2.55	40.69 ± 2.55

**Table 3** Statistical parameters for intra-laboratory reproducibility, in the case of method for determination of patulin in apple puree.

Sample name	Spiking level (µg/kg)	Mean value C <sub>Patulin</sub> (µg/kg)	SD(R) (µg/kg)	RSD(R) (%)	Reproducibility limit, R (µg/kg)	Expanded uncertainty (µg/kg)	Confidence interval (µg/kg)
Apple puree	20 (n = 6)	20.52	1.11	5.41	3.11	1.78	20.52 ± 1.78

reproducibility conditions RSD(R), in the case of method for determination of patulin in apple puree by high performance liquid chromatography, were within the provisions of the Regulation (EC) No. 401/2006 (EC regulation 2006a).

### 3.4 Limit of Detection (LOD) and Limit of Quantification (LOQ)

Limit of detection (LOD) represents concentration at which signal/noise ratio is higher than 3 (S/N >3). Limit of quantification (LOQ) is defined as being the lowest concentration of analyte which can be determined with an acceptable precision, in the conditions of a method of analysis, at a signal/noise ratio higher than 10 (S/N > 10). The obtained results were as follows: LOD = 3.11 µg/L (1.36 µg/kg) and LOQ = 10.40 µg/L (4.55 µg/kg).

Of the 17 commercial samples, none of them exceeded the maximum level of the CE Regulation (EC 1881/2006 (2006a) for patulin content (25 µg/kg), when 11 samples patulin was not detected. The mean

concentration of patulin, taking into consideration only the positive samples (samples with patulin concentration > LOQ), was 7.78 µg/kg and the incidence of positive samples was 35.29% (Table 4). Maximum patulin concentration of the analysed samples puree was 10.55 µg/kg (Fig. 5). The analysed puree samples contain apple puree (90%) and other ingredients (glucose-fructose syrup, sugar and citric acid), their dry matter being in the range 17.6 to 17.8 °Brix, and pH in the range 3.45 to 3.56.

Samples of apple puree prepared from two apple varieties intended for processing (*Jonathan*, *Florina*) fall under the maximum level of the EC Regulation 1881/2006 [13] for patulin content (25 µg/kg). Samples of apple puree prepared from apples obtained from conventional cultures have a lower patulin content in comparison with those obtained from uncertified organic cultures (Table 4).

In the case of samples of apple puree prepared from apple, *Jonathan* variety, the mean patulin concentration (taking into consideration only the positive samples)

**Table 4** Concentration of patulin in apple puree and apple and fruit puree.

Production method	Positive <sup>a</sup> /total	% Positives	Number of samples			Max. (µg/kg)	Mean ± SD positive samples (µg/kg)
			< LOD	< LOQ	4.62-10.55 (µg/kg)		
<b>Conventional</b>							
Commercial samples	6/17	35.29	11	0	6	10.55	7.78 ± 1.58
Apple puree from <i>Jonathan</i>	5/10	50	4	1	5	6.13	5.42 ± 0.54
Apple puree from <i>Florina</i>	5/12	41.66	5	2	5	5.19	4.93 ± 0.22
<b>Uncertified organic</b>							
Apple puree from <i>Jonathan</i>	5/10	50	3	2	5	7.37	6.20 ± 0.95
Apple puree from <i>Florina</i>	5/9	55.55	4	0	5	6.44	5.50 ± 0.60
<b>Organic</b>							
Blueberries and apple puree (for infants and young children)	0/5	0	5	0	0	-	-
Banana and apple puree (for infants and young children)	0/5	0	5	0	0	-	-

a. Samples with patulin concentration > LOQ;

b. Mean level was calculated using only positive sample.

### Assessment of Patulin Content in Apple Puree and Apple and Fruit Puree by High Performance Liquid Chromatography

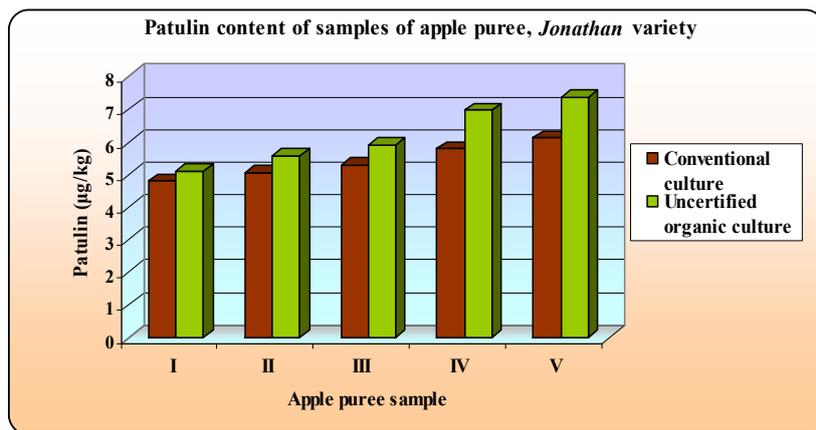


Fig. 5 Patulin content of samples of apple puree, *Jonathan* variety.

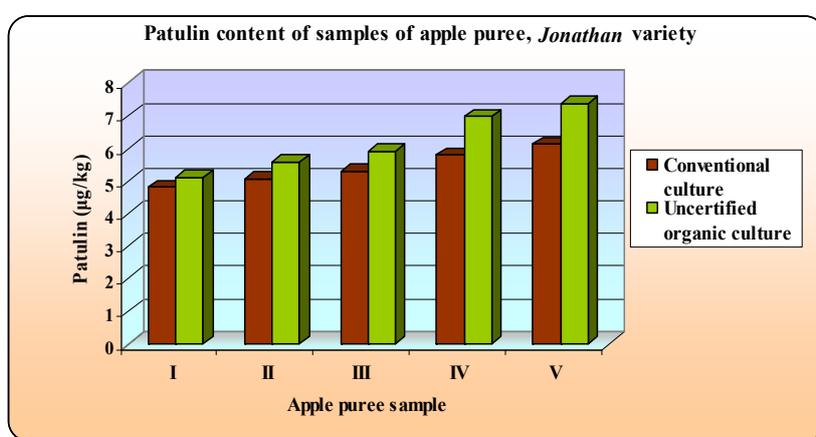


Fig. 6 Patulin content of samples of apple puree, *Jonathan* variety.

was 5.42 µg/kg, and the apple obtained from conventional culture, 6.20 µg/kg, in the case of those from uncertified organic culture.

In the case of 7 apple puree samples prepared from apple, *Jonathan* variety (4 samples of apple puree from apple obtained from conventional culture, 3 samples of apple puree from uncertified organic culture, respectively), patulin was not detected (Table 4). Concentration of patulin ranged from 4.81 to 7.37 µg/kg (Fig. 6), pH in the range of 3.37 to 3.51, and soluble dry matter in the range of 9.5 to 10.2 °Brix.

Based on the results obtained from the analysis of patulin concentration of puree samples, prepared from apple, *Jonathan* variety, there is a linear correlation, described by the equation  $y = -13.595x + 52.509$  (in case of apple from conventional culture) and  $y = -19.064x + 71.511$  (in case of apple from uncertified organic culture) respectively, while patulin

concentration being inversely proportional with the pH of apple puree (Figs. 7 and 8).

In the case of apple puree samples prepared from apple, *Florina* variety, patulin concentration ranged from 4.62 µg/kg to 6.44 µg/kg, being slightly lower than that recorded by samples of apple puree prepared from *Jonathan* variety (Fig. 9). From the samples studied, there were 41.66% positive samples in the case of puree prepared from conventional apple (maximum concentration being of 5.19 µg/kg), respectively 55.55% positive samples, in case of puree prepared from uncertified organic apple (maximum concentration being of 6.44 µg/kg). pH is ranged from 3.7 to 3.83.

Also, in case of *Florina* variety samples, a linear correlation has been observed between their patulin concentration and pH values, described by equations from Figs. 10 and 11.

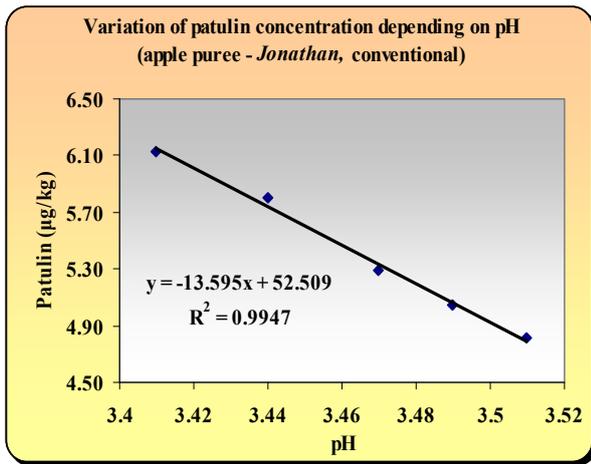


Fig. 7 Variation of patulin concentration depending on pH (apple puree—Jonathan, conventional).

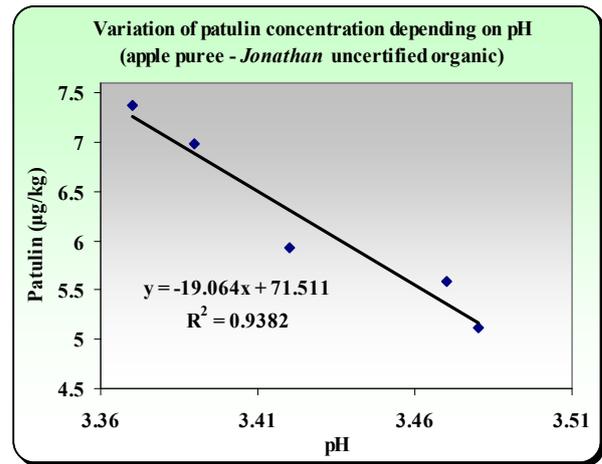


Fig. 8 Variation of patulin concentration depending on pH (apple puree—Jonathan, uncertified organic).

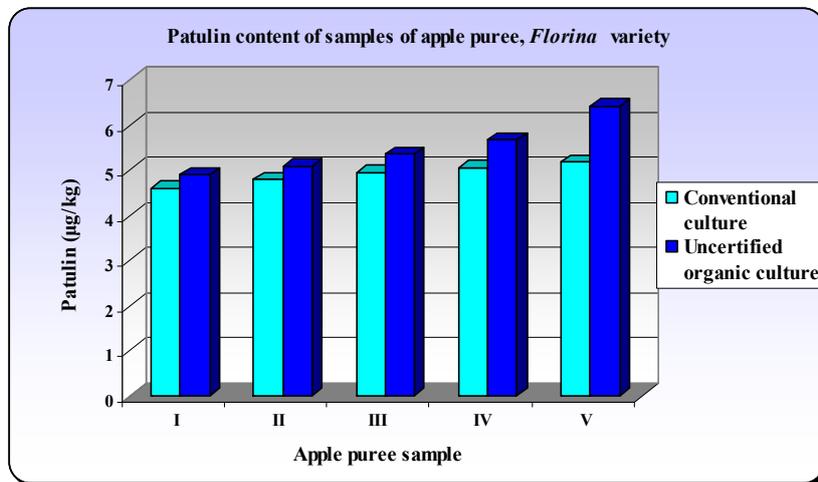


Fig. 9 Patulin content of samples of apple puree, Florina variety.

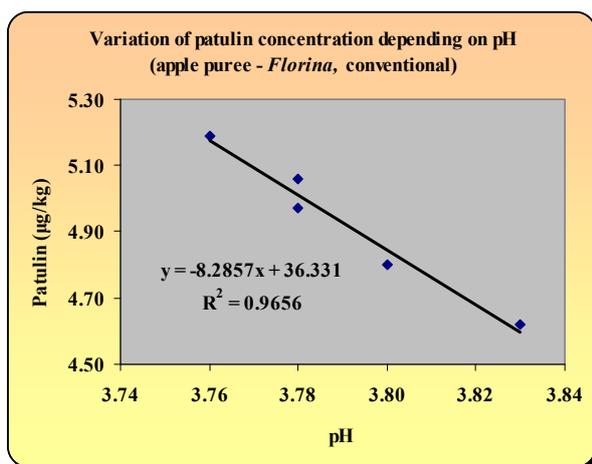


Fig. 10 Variation of patulin concentration depending on pH (apple puree—Florina, conventional).

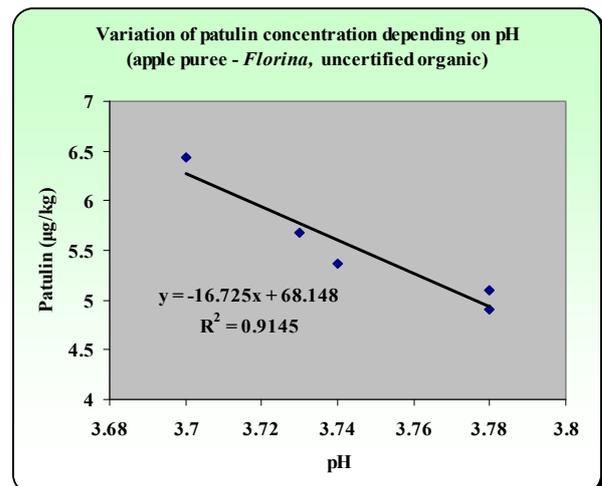


Fig.11 Variation of patulin concentration depending on pH (apple puree—Florina).

Patulin was not detected from the apple and fruit puree samples intended for consumption by infants and young children (Blueberries and apple puree, Banana and apple puree) which were analysed in this study.

#### 4. Conclusions

Within Human Nutrition Laboratory from the National R & D Institute for Food Bioresources—IBA Bucharest, an analytical method was developed and validated for determination of patulin in apple puree and apple and fruit puree by high performance liquid chromatography (HPLC-DAD). The proposed method has a good sensitivity (LOD = 1.36 µg/kg and LOQ = 4.55 µg/kg) and allows determination with a good precision of patulin concentration in samples of apple puree, apple and fruit puree respectively.

A number of 26 samples of apple puree (commercial samples, samples prepared within IBA Bucharest using apple intended for processing, from two varieties, both from the conventional culture and uncertified organic culture) were analysed using the proposed method with patulin concentration ranged from 4.62 µg/kg to 10.55 µg/kg (appreciably lower than the maximum level allowed by the present legislation—25 µg/kg), and in case of those 27 samples of apple puree, patulin was not detected. Also, in case of 5 samples of apple puree, patulin concentration was lower than the limit of quantification of the method (4.55 µg/kg).

Apple puree samples prepared from apple intended for processing, but from uncertified organic cultures, showed a patulin concentration slightly higher than of those from conventional ones, which were under the maximum level allowed by the legislation into force.

#### 5. Compliance with Ethical Standards

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#### Conflict of interest

The authors declare no conflict of interest.

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