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Original paper

Patulin analysis of some organic dried fruits samples by HPLC-DAD

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Abstract Fruits and vegetables present almost ideal conditions for the survival and development of many microorganisms. Berries are well known as fruits that have a very short shelf life after harvesting, mainly due to spoilage microorganisms, mostly fungi. Therefore, drying represents a usual technique used to preserve some of the nutritional and quality parameters of fresh berries, such as color, aroma, vitamin content, etc. In this study patulin concentration was determined for dried organic berries (bilberries and cranberries). Various pretreatment methods were used before drying. The results showed that patulin was not detected, regardless of the used drying or pretreatment process.

Keywords Organic, berry based products, mycotoxins, patulin

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Introduction

The most commonly berries harvested from the forest are bilberries, blackberries, cranberries and raspberries. Fresh berries are very sensitive and susceptible to fungal attack during post-harvest life. A wide spectrum of yeasts and fungi can often be found in various food products, where they can cause extensive damage and lead to great economic losses (MATEI & CORNEA [1]). Fungal infection often leads to food spoilage such as discoloration, rotting, off-flavors and disintegration of the food structure. The most important aspect involved in food spoilage by fungi is the formation of toxic secondary metabolites, namely mycotoxins (BLAGOJEV & al, ALI & al. [2, 3]), especially by *Fusarium* spp., *Aspergillus* spp. and *Penicillium* spp. (TOLOSA & al. [4]). Mycotoxins are metabolites of fungi, which can cause acute or chronical toxic effects (carcinogenic, mutagenic, teratogenic) on animals and humans. Patulin is a mycotoxin produced by a range of fungal species, generally *Penicillium, Aspergillus* and *Byssochlamys*, 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 contaminated commodities. Initially, patulin was considered as having therapeutic effect, as a result of its antibiotic properties. But in 1960s it was reclassified as mycotoxin because of its toxicity (PUEL & al. [5]).

The undertaken investigations have shown that patulin has mutagenic, neurotoxic, immunotoxic, genotoxic effects on rodents and different effects on gastro-intestinal tract, such as distension, ulceration and bleeding (HOPKINS [6]). 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 (MOAKE & al [7]).

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/kgbody weight/day, based on reproductive and carcinogenicitystudies and its toxicity (World Health Organization [8]).

Patulin appears mainly in moldy fruits. Although the presence of mold does not necessarily imply the presence of patulin in fruit, it indicates this possibility. In some circumstances, the inside development of molds may be due to insects or other invasions of healthy tissues, leading to the detection of patulin in fruit, which externally appears to be unaffected. However, patulin may occur in fruits after the storage under controlled atmosphere and exposure to the environmental conditions, with or without fruit pulp alteration. Washing fruit or removing the moldy tissue just before pressing will not remove patulin in the fruit, because it diffuses in the apparently healthy tissue (Codex Alimentarius Commission [9]).

According to IUPAC (International Union of Pure and Applied Chemistry), 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 (NIELSEN & SMEDSGAARD, CIEGLER & al [10, 11]). 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 (COLLIN & al [12]). The aim of the present study was to analyze some dried organic cranberries and organic bilberry press cake for patulin determination using HPLC-DAD method.

Materials and Methods

1. Materials

In this study, 31 samples of dried organic cranberries and 2 samples of organic bilberry press cake were analyzed for patulin determination. All the samples were received in lyophilised form. The cranberries were subjected to various pre-treatments before drying, thus fresh cranberries were cut in half and then an ultrasound (US) treatment was conducted at 21 kHz for 30 minutes in three liquid media:

- ✓ SA – 61.5% (w/w) sucrose solution
- ✓ STV – 30% (w/w) sucrose solution with 0.1% steviol glycosides addition
- ✓ T- 40% (w/w) trehalose

After US treatment the osmotic dehydration was performed for 72h at 40°C in these solutions (CUT_30US-SA, CUT_30US-STV, CUT_30US-T). As control, samples without ultrasound treatment were used, osmodehydrated with three different solutions (CUT-SA, CUT-STV, CUT-T). The samples were then stored at 10°C for 1, 2, 4 and 8 weeks (T1, T2, T4 and T8). The sample that was obtained immediately after osmodehydration was named T0.

Regarding the bilberry samples, bilberry press cake before hot air drying and bilberry press cake after hot air drying were analyzed. The description of the analyzed samples is presented in Table 1.

Table 1. The description of the analyzed samples		
No.	**Sample code**	**Sample description**
Samples T_0 (Cranberries)		
1.	T_0 - Fresh cranberries	Lyophilised fresh cranberries
2.	T_0 Cut_SA	Osmodehydratedcranberries in SA – 61.5% (w/w) sucrose solution.
3.	T_0 Cut_30_US_T	Ultrasounded (US) cranberries combined with osmotic dehydration in T - 40% (w/w) trehalose.
4.	T_0 Cut_US_30_SA	US cranberries combined with osmotic dehydration in SA – 61.5% (w/w) sucrose solution.
5.	T_0 Cut_US_30_STV	US cranberries combined with osmotic dehydration in STV – 30% (w/w) sucrose solution with 0.1% steviol glycosides addition.
6.	T_0 Cut_T	Osmodehydratedcranberries in T- 40% (w/w) trehalose
7.	T_0 Cut_STV	Osmodehydratedcranberries in STV – 30% (w/w) sucrose solution with 0.1% steviol glycosides addition
Samples T_1 (Cranberries)		
1.	T_1 Cut_SA	Osmodehydratedcranberries in SA – 61.5% (w/w) sucrose solution, and stored at 10°C for 1 week.
2.	T_1 Cut_US_T	US cranberries combined with osmotic dehydration in T- 40% (w/w) trehalose, and stored at 10°C for 1 week.
3.	T_1 Cut_T	Osmodehydratedcranberries in T- 40% (w/w) trehalose, and stored at 10°C for 1 week.
4.	T_1 Cut_US_STV	US cranberries combined with osmotic dehydration in STV – 30% (w/w) sucrose solution with 0.1% steviol glycosides addition, and stored at 10°C for 1 week.
5.	T_1 Cut_STV	Osmodehydratedcranberries in STV – 30% (w/w) sucrose solution with 0.1% steviol glycosides addition, and stored at 10°C for 1 week.
6.	T_1 Cut_US_SA	US cranberries combined with osmotic dehydration in SA – 61.5% (w/w) sucrose solution, and stored at 10°C for 1 week.
Samples T_2 (Cranberries)		
1.	T_2 Cut_SA	Osmodehydratedcranberries in SA – 61.5% (w/w) sucrose solution, stored at 10°C for 2 weeks.
2.	T_2 Cut_T	Osmodehydratedcranberries in T- 40% (w/w) trehalose, stored at 10°C for 2 weeks.
3.	T_2 Cut_STV	Osmodehydratedcranberries in STV – 30% (w/w) sucrose solution with 0.1% steviol glycosides addition, stored at 10°C for 2 weeks.
4.	T_2 Cut_US_30_STV	US cranberries combined with osmotic dehydration in STV – 30% (w/w) sucrose solution with 0.1% steviol glycosides addition, stored at 10°C for 2 weeks.

5.	T_2 Cut_US_30_T	US cranberries combined with osmotic dehydration in T- 40% (w/w) trehalose, stored at 10°C for 2 weeks.
6.	T_2 Cut_US_30_SA	US cranberries combined with osmotic dehydration in SA – 61.5% (w/w) sucrose solution, stored at 10°C for 2 weeks.
Samples T_4 (Cranberries)		
1.	T_4 Cut_STV	Osmodehydratedcranberries in STV – 30% (w/w) sucrose solution with 0.1% steviol glycosides addition, stored at 10°C for 4 weeks.
2.	T_4 Cut_SA	Osmodehydratedcranberries in SA – 61.5% (w/w) sucrose solution, stored at 10°C for 4 weeks.
3.	T_4 Cut_T	Osmodehydratedcranberries in T- 40% trehalose, stored at 10°C for 4 weeks.
4.	T_4 Cut_US_30_STV	US cranberries combined with osmotic dehydration in STV – 30% (w/w) sucrose solution with 0.1% steviol glycosides addition, stored at 10°C for 4 weeks.
5.	T_4 Cut_US_30_SA	US cranberries combined with osmotic dehydration in SA – 61.5% (w/w) sucrose solution, stored at 10°C for 4 weeks.
6.	T_4 Cut_US_30_T	US cranberries combined with osmotic dehydration in T- 40% (w/w) trehalose, stored at 10°C for 4 weeks.
Samples T_8 (Cranberries)		
1.	T_8 Cut_T	Osmodehydratedcranberries in T- 40% (w/w) trehalose, stored at 10°C for 8 weeks.
2.	T_8 Cut_SA	Osmodehydratedcranberries in SA – 61.5% (w/w) sucrose solution, stored at 10°C for 8 weeks.
3.	T_8 Cut_STV	Osmodehydratedcranberries in STV – 30% (w/w) sucrose solution with 0.1% steviol glycosides addition, stored at 10°C for 8 weeks.
4.	T_8 Cut_US_30_T	US cranberries combined with osmotic dehydration in T- 40% (w/w) trehalose, stored at 10°C for 8 weeks.
5.	T_8 Cut_US_30_SA	US cranberries combined with osmotic dehydration in SA – 61.5% (w/w) sucrose solution, stored at 10°C for 8 weeks.
6.	T_8 Cut_US_30_STV	US cranberries combined with osmotic dehydration in STV – 30% (w/w) sucrose solution with 0.1% steviol glycosides addition, stored at 10°C for 8 weeks.
Bilberry press cake		
1.	Bilberry press cake	Fresh bilbery press cake (before drying)
2.	Hot air dried bilberry press cake	Hot air dried bilberry press cake

2. Method – Determination of Patulin by HPLC-DAD

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, reference material (5 mg, purity = 99.5%) was obtained from Sigma-Aldrich (code 32759). C.U. Patulin MycoSep® 228 AflaPat (Romer Labs) columns were used for cleaning of sample extract.

Sample Preparation

Samples taken in the study were milled using the Retsch mill. In a 50 mL centrifuge tube weigh to the nearest 0.0001 g, 0.5 sample and add 20 mL of ultrapure water. Then, the sample was vortexed (Vortex Heidolph) for 90 min and centrifuged (Centrifuge Eppendorf 5804R, Germany) at 9000 rpm, for 50 min at 5ºC. 4 mL of the supernatant and 21 mL acetonitrile were introduced into a brown vial and vortex for 15 min to extract the patulin in acetonitrile. The sample extract was purified using a C.U. Patulin MycoSep® 228 AflaPat column. The purified sample extract (4 mL) was evaporated near dryness under a stream of nitrogen at 40°C. Residue was redissolved in 1 mL of solution acetonitrile: water (pH = 4) = 10:90 (v/v) and was analysed by HPLC.

Parameters and conditions of HPLC-DAD method for determination of patulin

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, ChromQuest 4.2 software for data acquisition and data processing). The separation was performed at 25°C, on a C18 Hypersil GOLD (150 x 4 mm, 5 µm) with a Hypersil Gold guard column (10 x 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.

Results and Discussions

Calibration curve

For the calibration curve, patulin standard in crystalline form (5 mg) was used. The standard 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 (Figure 1), based on equation 1:

$$\rho_{pat} = A_{max} \times M \times 100 / \varepsilon \times \delta \quad [1]$$

where:

ρ_{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)

ε – relative molar absorption coefficient of patulin in ethanol (in this case 1460 m^2/mol, according to AOAC Official Methods, 1995, Natural Toxins, Patulin, 49.6.01. C(d))

δ – the optical path length of quartz cuvette in cm

In the case of the stock solution Amax = 0.9479, and concentration of solution was ρ_{pat}= 10.00 µg/mL.

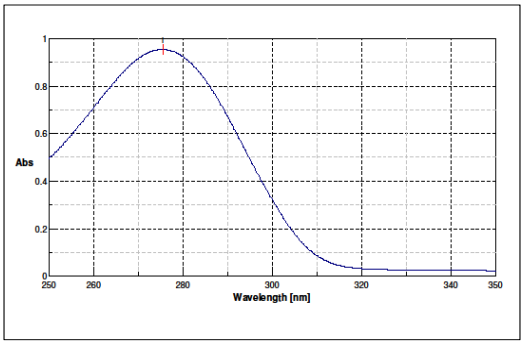
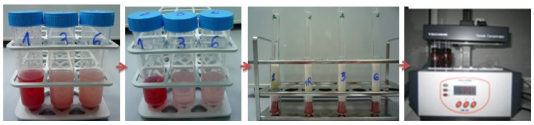


Figure 1. Spectrum of patulin alcoholic solution

Seven patulin standard levels (with three replicate injections from each level), in concentration range from 6.25 µg/L to 400 µg/L have been used for the calibration curve. Obtained linear regression values: linear regression equation: y = 696752x-430.897; regression coefficient R2 = 0.999994. The limit of detection of the method of analysis is 3.49 µg/L, and the limit of quantification is 11.64 µg/L.

In Figure 2 are presented aspects of the method of analysis in case of T_0 samples (samples after vortexing for 90 min, respectively, after centrifugation at 5°C for 50 min; purification of sample extract using C.U. Patulin MycoSep® 228 AflaPat columns and evaporation of the purified sample extract under nitrogen at 40°C).



(**a**) (**b**) (**c**) (**d**)

Figure 2. Aspects of the patulin determination method (Sample: 1-Fresh cranberries; 2-T_0 Cut_SA; 3-T_0 Cut_30_US_T) (**a**) – samples after vortexing for 90 min; (**b**) – samples after centrifugation at 5°C for 50 min; (**c**) – purification of sample extract using C.U. Patulin MycoSep® 228 AflaPat columns; (**d**) – evaporation of sample extract under nitrogen at 40°C

Following HPLC-DAD analysis of the purified and concentrated extracts of the samples taken in the study, patulin was not detected. In Figure 3 the chromatogram and test report for a patulin standard (certified material reference) with the concentration of 0.04947 µg/mL is presented, the retention time of patulin being 4.600 min.

The rest of the samples were analyzed identically, the results being presented in Table 2. An example can be seen in Figure 4 where the superimposed chromatograms of the fresh cranberries samples (Fresh_1_08.03.2017 and Fresh_2_08.03.2017) and the patulin standard (certified material reference) are presented.

In Table 2, the results for patulin determination for all tested samples are presented.

Table 2. Results of patulin determination in dried organic cranberries and bilberries

No.	Sample code	Patulin (µg/L)
Samples T_0 (Cranberries)		
1.	T_0 - Fresh cranberries	N.d.*
2.	T_0 Cut_SA	N.d.*
3.	T_0 Cut_30_US_T	N.d.*
4.	T_0 Cut_US_30_SA	N.d.*
5.	T_0 Cut_US_30_STV	N.d.*
6.	T_0 Cut_T	N.d.*
7.	T_0 Cut_STV	N.d.*
Samples T_1 (Cranberries)		
1.	T_1 Cut_SA	N.d.*
2.	T_1 Cut_US_T	N.d.*
3.	T_1 Cut_T	N.d.*
4.	T_1 Cut_US_STV	N.d.*
5.	T_1 Cut_STV	N.d.*
6.	T_1 Cut_US_SA	N.d.*
Samples T_2 (Cranberries)		
1.	T_2 Cut_SA	N.d.*
2.	T_2 Cut_T	N.d.*
3.	T_2 Cut_STV	N.d.*
4.	T_2 Cut_US_30_STV	N.d.*
5.	T_2 Cut_US_30_T	N.d.*
6.	T_2 Cut_US_30_SA	N.d.*
Samples T_4 (Cranberries)		
1.	T_4 Cut_STV	N.d.*
2.	T_4 Cut_SA	N.d.*
3.	T_4 Cut_T	N.d.*
4.	T_4 Cut_US_30_STV	N.d.*
5.	T_4 Cut_US_30_SA	N.d.*
6.	T_4 Cut_US_30_T	N.d.*
Samples T_8 (Cranberries)		
1.	T_8 Cut_T	N.d.*
2.	T_8 Cut_SA	N.d.*
3.	T_8 Cut_STV	N.d.*
4.	T_8 Cut_US_30_T	N.d.*
5.	T_8 Cut_US_30_SA	N.d.*
6.	T_8 Cut_US_30_STV	N.d.*
Bilberry press cake		
1.	Bilberry press cake	N.d.*
2.	Hot air dried bilberry press cake	N.d.*

*< LOD (LOD = 3.49 µg/L)

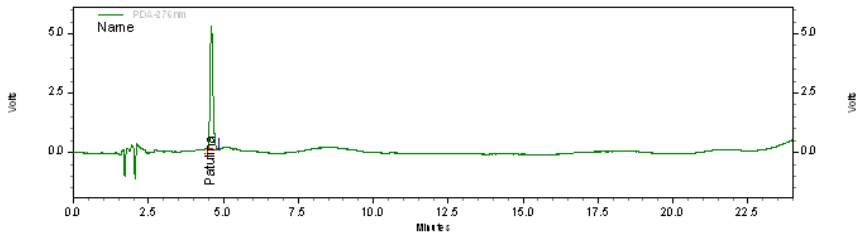


Figure 3. Chromatogram of the patulin standard (certified material reference) with the concentration of 0.04947 µg/mL (retention time = 4.600 min)

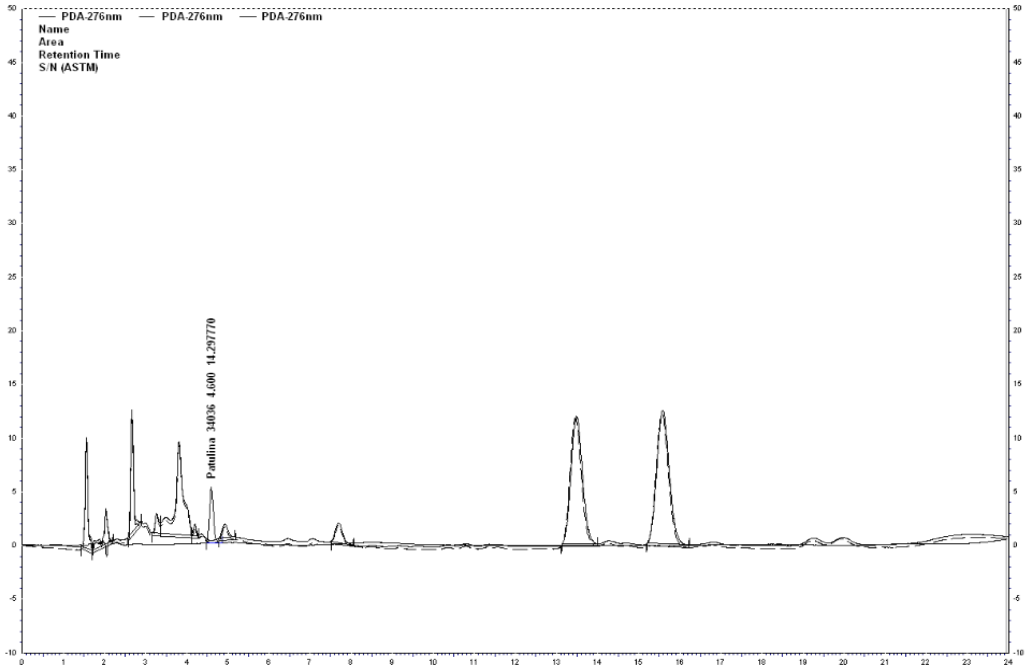


Figure 4. Superimposed chromatograms of the fresh cranberries samples (Fresh_1_08.03.2017 and Fresh_2_08.03.2017) and the patulin standard (certified material reference), respectively.

Several studies are in accordance with the results obtained in this study. VACLAVIKOVA & al. [13] analyzed several fresh fruits and juices based on berries using UHPLC-MS/MS analytical procedure. In their study, patulin was not detected for various berries such as strawberries (fresh fruits and juice), grape vines (fresh fruits and juice) and fresh fruits of raspberries, blueberries, blackberries and sour cherries. SADOK & al. [14] also analyzed fresh strawberries using HPLC-DAD method and their results showed that patulin was below the limit of detection for their samples. Another study performed by KATAOKA & al. [15] targeted various dried fruits for patulin determination using LC-MS method. It was concluded that patulin was not detectable in any of the dried analyzed samples (apple, apricot, kiwi, prune, pineapple, papaya, mango and fig).

Conclusions

In this study, patulin was determined for 31 samples of dried organic cranberries and 2 samples of organic bilberry press cake. Following HPLC-DAD analysis of the purified and concentrated extracts of the studied samples, patulin was not detected in any of the dried cranberries and bilberries samples, regardless of the used drying method.

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