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## Use of pulsed-high hydrostatic pressure treatment to decrease patulin in apple juice

M.D. Avsaroglu<sup>a\*</sup>, F. Bozoglu<sup>b</sup>, H. Alpas<sup>b</sup>, A. Largeteau<sup>c</sup> and G. Demazeau<sup>c</sup>

<sup>a</sup>Department of Agricultural Biotechnology, Ahi Evran University, Kirsehir, Turkey; <sup>b</sup>Department of Food Engineering, Middle East Technical University, Ankara, Turkey; <sup>c</sup>ICMCB-CNRS, Université Bordeaux I, Bordeaux, France

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This study was aimed at reducing patulin content of apple juice using a non-thermal method, namely pulsed-high hydrostatic pressure (p-HHP). Commercially available clear apple juice was contaminated artificially with different concentrations of patulin (5, 50 and 100 ppb). Then, the samples were processed 5 min at different pressure treatments (300–500 MPa) in combination with different temperatures (30–50°C) and pulses (6 pulses × 50 s and 2 pulses × 150 s). To compare the impact of pulses, single pulse of high hydrostatic pressure (HHP) treatment was also applied with the same pressure/temperature combinations and holding time. Results indicated that pressure treatment in combination with mild heat and pulses reduced the levels of patulin in clear apple juice up to 62.11%. However, reduction rates did not follow a regular pattern. p-HHP was found to be more effective in low patulin concentrations, whereas HHP was more effective for high patulin concentrations. To the best of our knowledge, this is the first study using p-HHP to investigate the reduction of patulin content in apple juice.

**Keywords:** patulin; apple juice; pulsed-high hydrostatic pressure

### 1. Introduction

Patulin (4-hydroxy-4H-furo[3,2c]pyran,2[6H]-one) is a mycotoxin which is produced as a secondary metabolite by mould species, particularly by *Penicillium*, *Aspergillus*, *Byssoschlamys* species. Pre-harvest and post-harvest occurrences of patulin have been shown in several fruits especially in apple and apple products.

Toxicity of patulin varies as mutagenic, immunogenic and neurotoxic as shown with animal studies.[1] Due to these toxic effects of patulin, contaminated foods possess potential health risks, and authorities have regulated tolerable daily intake of patulin. For adults, it was limited to 50 µg/kg patulin in apple-containing foods and since it is more hazardous to infants and children than adults, 10 µg/kg tolerable daily intake was regulated in infant foods by the EU Commission.[2]

Not only microorganisms, but also their toxic metabolites must be eliminated from foods so that food safety can be assured. For this reason, patulin contamination in apple products must be reduced to lower limits than regulated limits. Many studies have been made in order to reduce the toxin during apple juice production processes.[3,4] These include physical treatments such as filtration of apple juice,[5] chemical treatments such as ammoniation [3] and biological

\*Corresponding author. Email: [dilek.avsaroglu@gmail.com](mailto:dilek.avsaroglu@gmail.com)

degradation of patulin by using yeast cells.[6] On the other hand, hurdle technologies have also been studied for this aim. Heat treatment,[7] UV processing,[8] irradiation [9] and pulsed light application [10] were applied to reduce patulin contamination in apple juice. All these attempts resulted in the reduction of patulin, but they had a considerable variation between different applications or studies with the same application.

High hydrostatic pressure (HHP) treatment of foods is a “non-thermal” food preservation technique, which improves microbiological quality of foods and supplies fresh-like foods to consumers.[11] The main advantage of HHP treatment is that unlike thermal treatment, organoleptic properties of foods are not affected while high microbiological and nutritious quality is achieved.[12] It has been the subject of research and development in the world, and within a very short time it was led to the submission of various products on the market.[13]

There are research studies to reduce microbial toxins in foods. In a research study, Bruna et al.[14] exhibited that at 800 MPa, patulin reduced up to 62% and 23% in apple juices and concentrates, respectively. Tokusoglu et al.[15] stated a decrease in levels of citrinin up to 56% in black olive oils by HHP application. Bacterial toxins were also inactivated by HHP.[16]

There has not been a prominent food preservation method to solve patulin problem fundamentally in apple juice and products. In this respect, HHP application for patulin reduction studies should be enhanced to clarify the effect of pressure as a food preservation method, because there were successful studies in order to reduce toxic compounds by HHP.[14–16] By HHP treatment, patulin levels could be reduced, and also patulin producers and other pathogens could be inactivated. For example, Bruna et al.[14] achieved up to 62% reduction of patulin at 800 MPa in apple juice, whereas Palou et al.[17] inactivated *Byssoschlamys nivea* spores at 689 MPa by oscillatory HHP at 60°C. Thus, pressure treatments higher than 700 MPa ensures patulin producer’s spore inactivation.

The aim of this study was to investigate the effect of HHP and pulsed-high hydrostatic pressure (p-HHP) on patulin reduction in commercial clear apple juice and to compare with continuous HHP in order to evaluate efficiencies of both pressure applications.

## 2. Materials and methods

### 2.1. Sample preparation

Patulin ( $\geq 98\%$  TLC, Sigma Life Sciences, USA) stock solution (50 ppm) was prepared according to Gökmen and Acar.[18] This stock solution was kept in the dark at  $-20^{\circ}\text{C}$ .[19] Clear apple juice was purchased from local supermarkets. It was a squeezed, pasteurized and aseptically packed commercial product. Samples were prepared freshly before each pressure treatment by diluting the stock solution with the apple juice. The diluted sample concentrations of patulin were 5, 50 and 100 ppb. About 20 mL of apple juice without artificial contamination was kept in the dark at  $-20^{\circ}\text{C}$  and used as a control in the high-performance liquid chromatography (HPLC) analyses.

Samples were transferred into cryovial tubes (2 mL each) and sealed so that there were no air bubbles in the tubes. Each sample was prepared in two parallels and pressure processed to both parallels at the same time.

### 2.2. Pressure applications

In this study, two different pressure processes were applied to the samples. One was HHP application, and the other was p-HHP application.

### 2.2.1. HHP applications

HHP process was performed with 760.0118-type pressure equipment supplied by SITEC-Sieber Engineering AG, Zurich, Switzerland. The vessel had a volume of 100 mL with inner diameter 24 mm and length 153 mm. A built-in heating/cooling system (Huber Circulation Thermostat, Offenburg, Germany) was used to maintain and control required temperature that is measured by a thermocouple K-type in the vessel. Pressure-transmitting medium was distilled water filled in the vessel. Pressure treatments were applied at 300, 400 and 500 MPa, and 20°C, 30°C, 40°C and 50°C were used for each pressure treatment. Pressure-holding time of each treatment was 5 min excluding pressure increase and release times (< 20 s). All samples were stored in a water bath at the corresponding temperatures for 5 min to set the temperature prior to HHP process. Pressurized samples were kept in the dark at -20°C until toxin extractions. Control apple juices were not pressure treated.

### 2.2.2. p-HHP applications

p-HHP process of the samples was carried out using a computer-controlled high pressure unit with 3 L sample compartment. It was capable of operating at up to 800 MPa (designed by NFM-Technologies, Le Creusot, France and FRAMATOME, Paris, France, marketed by CLEXTRAL, Firminy, France). Pressure-transmitting medium was distilled water. Pressure and temperature were measured using sensors inside and outside the high pressure vessel. The samples were pressurized at 300, 400, and 500 MPa for a total holding time of 5 min. The pressure-holding time reported in this study did not include the process come-up or depressurization times. Of the temperatures, 30°C, 40°C and 50°C were used for each pulse pressure treatment. During pressurization, due to adiabatic heat determined using a K-type thermocouple, 20°C temperature was not included in p-HHP applications. Two different pulses were applied: 2 pulses  $\times$  150 s and 6 pulses  $\times$  50 s. Samples were handled as described above. Control apple juices were not pulse pressure treated.

## 2.3. Patulin extraction from samples

Patulin was extracted from pressure treated samples by using molecularly imprinted polymer columns (Easimip-patulin, R-Biopharm Rhône Ltd.). By following the manufacturer's instructions, briefly, samples were diluted 1:1 with 2% acetic acid. Columns were conditioned with 2 mL of 100% acetonitrile and then with 1 mL of distilled water. Diluted samples were passed through the columns slowly (0.5 mL/min). Afterwards, 1 mL of 1% sodium bicarbonate, 2 mL of distilled water and 1 mL of 100% diethyl ether were passed (1 mL/min) through the columns, respectively. Patulin was eluted from the columns by using 2 mL of 100% ethyl acetate. The samples were dried by evaporation with nitrogen gas. About 1 mL of 0.1% formic acid was used to reconstitute patulin extracts. Patulin extracts were kept in the dark at -20°C until HPLC analyses.

## 2.4. HPLC analyses

Quantifications of patulin extracts were performed with HPLC system Agilent 1100 (Waldbronn, Germany) consisting of a quaternary pump, an auto sampler, a diode array detector and a temperature-controlled column oven. An isocratic mixture of 10 mM aqueous formic acid solution and acetonitrile (90:10, v/v) were used as the mobile phase at a flow rate of 1 mL/min at

25°C. Data acquisition was performed acquiring chromatograms at a detection wavelength of 285 nm.

The quantities of patulin were determined by using calibration curve ( $R^2 = 0.9997$ ). Standards were prepared by diluting the patulin stock solution to concentrations of 100, 500 and 1000 ppb with mobile phase. In order to calculate patulin concentrations, peak areas of the sample and those of the standard solutions were compared.

## 2.5. Statistical analyses

The results were reported as mean patulin reduction (%) values  $\pm$  standard error. SPSS 16 for Windows (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. One-way analyses of variance and Tukey's HSD ( $p < 0.05$ ) were applied to test the significance of difference between pressure treatments and patulin concentrations.

## 3. Results and discussion

### 3.1. Effect of HHP applications

In this study, different pressure levels (300–500 MPa) in combination with temperature (20–50°C) were applied to artificially contaminated clear apple juice. The effects of combined treatments are presented in Table 1. Patulin decrease was ranged from 0% to 51.16%. Tokusoglu et al.[15] found that citrinin levels reduced in black table olives at 250 MPa/35°C for 1 min. Comparing with our results, it can be concluded that patulin reduction can be obtained at higher pressure levels than that of citrinin. This finding is also the case that HHP affects microorganisms and their spores at different pressure levels.

As can be observed from standard errors in Table 1, patulin reduction was variable between parallels. There were not a linear decrease in patulin concentrations for different pressure/temperature applications, and the highest reduction was obtained at 400 MPa/30°C. There was not any statistically significant difference between different pressure applications including 400 MPa/30°C ( $p > 0.05$ ). Bruna et al.[14] studied the reduction of patulin in apple juice by high pressure application (300, 500 and 800 MPa) at room temperature for 1 h. They obtained 42%, 53% and 62% reductions, respectively. Authors indicated that the exposure time of pressure was

Table 1. % Reduction of patulin by HHP application.

	20°C	30°C	40°C	50°C
5 ppb				
300 MPa	0.00 $\pm$ 0.00	11.53 $\pm$ 16.53	16.39 $\pm$ 1.94	5.69 $\pm$ 16.53
400 MPa	6.67 $\pm$ 13.61	22.22 $\pm$ 15.56	8.61 $\pm$ 5.83	21.25 $\pm$ 10.69
500 MPa	5.70 $\pm$ 0.97*	0.00 $\pm$ 0.00	28.06 $\pm$ 9.72	35.16 $\pm$ 9.04
50 ppb				
300 MPa	16.71 $\pm$ 14.39	37.15 $\pm$ 4.54	26.55 $\pm$ 13.63	18.98 $\pm$ 7.57
400 MPa	11.41 $\pm$ 12.87	51.16 $\pm$ 10.22	20.88 $\pm$ 4.16	10.28 $\pm$ 4.16
500 MPa	14.06 $\pm$ 4.92	0.00 $\pm$ 0.00	11.79 $\pm$ 12.49	35.64 $\pm$ 16.66
100 ppb				
300 MPa	5.72 $\pm$ 1.35	30.08 $\pm$ 13.53	21.28 $\pm$ 0.23	27.37 $\pm$ 16.24
400 MPa	10.45 $\pm$ 12.41	37.52 $\pm$ 18.72	25.79 $\pm$ 7.89	29.17 $\pm$ 11.28
500 MPa	24.66 $\pm$ 0.90*	10.67 $\pm$ 14.88	24.66 $\pm$ 4.96	24.89 $\pm$ 9.25

\*Values in the same column are significantly different at  $p < 0.05$ .

a parameter to reach higher reductions of patulin. Our results were parallel to those obtained by Bruna et al.[14] if pressure-holding times were considered. For 5 min pressure application, in our results, 5.7% (5 ppb), 14.06% (50 ppb) and 24.66% (100 ppb) reductions were observed at 500 MPa/20°C.

For different initial patulin concentrations, patulin reductions starting with 5 and 100 ppb were statistically significant ( $p < 0.05$ ) at 500 MPa/20°C. Figure 1 exhibits the reduction of 100 ppb patulin by this application in comparison to control sample. Thus, HHP application is more effective for high patulin concentrations ( $> 5$  ppb). When the applied temperatures were taken into account, it was concluded that pressure rather than temperature was responsible for reduction of patulin. Bruna et al.[14] have indicated that when higher temperature (50°C) was applied, the reduction of patulin was achieved in shorter time. Margosch et al.[16] studied different bacterial toxins, loss of immuno-reactivity of staphylococcal enterotoxins A–E and *Escherichia coli* enterotoxin STa were reported, and cholera toxin in latex agglutination was lost at 800 MPa/80°C at different pressurizing times. These toxins were peptide/polypeptide molecules and heat stable and/or labile. The authors discussed that the loss of cytotoxicity was mainly due to changes in tertiary structure. In contrast, same study exhibited the increase in *Bacillus cereus* HBL toxin's immune reactivity at 800 MPa/30°C, which was explained with dissociation of HBL complex resulting better accessibility for the antibodies.[16] It is known that HHP primarily effects hydrophobic and electrostatic interactions of protein molecules and covalent bonds are not affected.[20] In the case of patulin, it is a much smaller molecule than those bacterial toxins and composed of only covalent bonding. In this respect, HHP application can be considered as an ineffective approach for patulin reduction studies. However, the reduction of patulin resulted in our findings can be explained by adduct formation of patulin with other compounds in apple juice environment. These compounds possess sulphhydryl groups such as cysteine and glutathione.[21] Bruna et al.[14] have reported that by the addition of sodium metabisulfite in apple concentrate; pressure effect on patulin was more relevant. Merkulow and Ludwig [22] were also reported that the formation of cysteine–patulin adduct was increased by the application of different pressure/temperature combinations. Thus, this is probably the reason why our results did not follow a regular pattern, since sulphhydryl group bearing molecules were not inspected in the clear apple juice used.

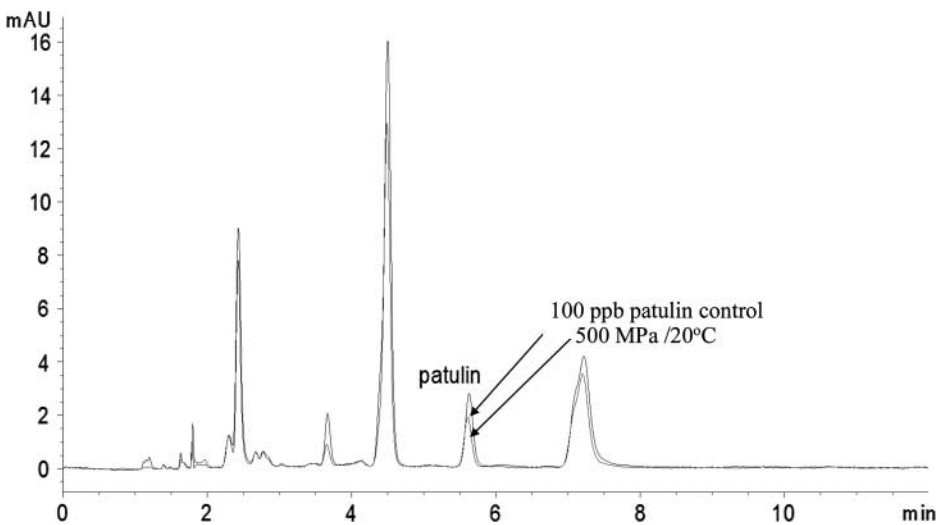


Figure 1. HPLC chromatogram of 100 ppb patulin control and 500 MPa/20°C application to this concentration.

### 3.2. Effect of p-HHP applications

In p-HHP application, besides pressure and temperature, another parameter was added to the experiment, namely pulses. Rather than applying one cycle or continuous pressure treatment for 5 min, pressure was subjected with pulses to the samples with a total of 5 min holding time. Two different pulses were used in this study: 6 pulses  $\times$  50 s and 2 pulses  $\times$  150 s.

Table 2 shows the reduction results of 6 pulses  $\times$  50 s applications. Overall, the reductions were changed between 0% and 62.11%. A remarkable point was that patulin reduction occurred in a concentration-dependent manner. For 5 ppb initial patulin concentration of clear apple juice, reductions were between 12.27% and 62.11%. On the other hand, 6.92–29.16% reductions were achieved for 50 ppb initial concentrations and 0–18.47% reductions were achieved for 100 ppb initial concentrations. Also, the difference in reduction levels obtained from p-HHP/temperature treatments applied to 5 ppb initial patulin concentration were found to be statistically significant ( $p < 0.05$ ).

The second p-HHP application (2 pulses  $\times$  150 s) also reduced patulin in clear apple juice (Table 3). Reduction patterns were similar to those obtained from 6 pulses  $\times$  50 s applications. The results exhibited 0–45.49% reductions for 5 ppb, 1.25–19.38% for 50 ppb and 0–17.69% for 100 ppb initial patulin concentrations. Among all p-HHP/temperature treatments using 2 pulses  $\times$  150 s, there were no statistically significant ( $p < 0.05$ ) difference. The highest reduction was 45.49% at 300 MPa/50°C. When pressure was increased to 500 MPa, the reduction rates were reduced or not changed significantly ( $p < 0.05$ ). On the other hand, increasing temperature from 30°C to 50°C gave conflicting results.

In general, results obtained from p-HHP treatments revealed that patulin reductions were initial concentration dependent. In both 6 pulses  $\times$  50 s and 2 pulses  $\times$  150 s applications at lower initial patulin levels, the reductions were higher.

To the best of our knowledge, this is the first study to determine the effect of p-HHP on patulin reduction in clear apple juice. On the other hand, oscillatory- or p-HHP researches in food science were studied in order to understand its efficiency. Palou et al.[17] were evaluated oscillatory HHP treatment effects on *B. nivea* ascospores in diluted apple and cranberry juice concentrates. They reported that 3 or 5 cycles of 689 MPa pressure with 1 s holding times at 60°C application inactivated the spores in both fruit juices. *B. nivea* spores are heat resistant and produce patulin

Table 2. % Reduction of patulin by p-HHP application (6 pulse  $\times$  50 s).

	30°C	40°C	50°C
5 ppb			
300 MPa	30.81 $\pm$ 3.75	12.79 $\pm$ 6.75	62.11 $\pm$ 8.31 <sup>a</sup>
400 MPa	40.80 $\pm$ 16.74	20.32 $\pm$ 11.68	37.19 $\pm$ 1.56 <sup>a</sup>
500 MPa	25.81 $\pm$ 1.75 <sup>a</sup>	12.27 $\pm$ 2.08 <sup>ab</sup>	31.48 $\pm$ 5.71 <sup>a</sup>
50 ppb			
300 MPa	15.06 $\pm$ 11.62	16.62 $\pm$ 1.20	21.17 $\pm$ 4.65 <sup>b</sup>
400 MPa	19.13 $\pm$ 1.29	15.27 $\pm$ 4.15	22.23 $\pm$ 1.00 <sup>ab</sup>
500 MPa	29.17 $\pm$ 4.77 <sup>a</sup>	18.47 $\pm$ 1.65 <sup>a</sup>	21.02 $\pm$ 3.40 <sup>ab</sup>
100 ppb			
300 MPa	7.91 $\pm$ 9.46	0.00 $\pm$ 0.00	6.91 $\pm$ 3.59 <sup>b</sup>
400 MPa	16.67 $\pm$ 5.70	0.56 $\pm$ 3.46	18.47 $\pm$ 4.17 <sup>b</sup>
500 MPa	2.97 $\pm$ 3.10 <sup>b</sup>	0.00 $\pm$ 0.00 <sup>b</sup>	0.00 $\pm$ 0.00 <sup>b</sup>

Note: Values with different letters in the same column are significantly different at  $p < 0.05$ .

Table 3. % Reduction of patulin by p-HHP application (2 pulse  $\times$  150 s).

	30°C	40°C	50°C
5 ppb			
300 MPa	17.57 $\pm$ 4.00	42.64 $\pm$ 23.10	45.49 $\pm$ 24.40
400 MPa	18.82 $\pm$ 1.75	41.34 $\pm$ 7.27	23.17 $\pm$ 11.94
500 MPa	18.57 $\pm$ 0.01	0.00 $\pm$ 0.00	28.10 $\pm$ 11.16
50 ppb			
300 MPa	19.38 $\pm$ 6.61	15.57 $\pm$ 1.65	1.26 $\pm$ 3.45
400 MPa	13.77 $\pm$ 12.82	18.97 $\pm$ 1.35	8.76 $\pm$ 5.86
500 MPa	17.04 $\pm$ 7.15	17.52 $\pm$ 0.30	7.51 $\pm$ 6.41
100 ppb			
300 MPa	17.69 $\pm$ 6.36	2.93 $\pm$ 6.83	6.76 $\pm$ 6.23
400 MPa	15.17 $\pm$ 0.95	5.83 $\pm$ 14.12	9.71 $\pm$ 8.39
500 MPa	14.93 $\pm$ 10.80	0.11 $\pm$ 14.72	0.00 $\pm$ 0.00

in pasteurized fruit juices after spore germination.[23] By using p-HHP, it can be discussed that patulin reduction can be achieved together with patulin producer mould's spores. However, the most efficient pressure/temperature/pulse combination must be further investigated. Oscillatory pressure application was found to increase the effectiveness of HHP processing for inactivation of *Zygosaccharomyces bailii* at 276 MPa pressure.[24] Different bacterial inactivations were also demonstrated by using oscillatory- or p-HHP.[25–27]

### 3.3. Comparison of HHP and p-HHP applications

High pressure processing of foods has a great potential to assure food safety and to fulfil the consumer demand for better sensory and nutritional quality of foods. Another aspect is to apply p-HHP rather than to use continuous HHP. Efficiencies of p-HHP over HHP treatments have been investigated by several authors and found to be superior with certain treatment conditions.[17,26,27]

In our study, both HHP and p-HHP applications exhibited patulin reduction but results did not follow a regular pattern. The highest reductions were 51.16% (400 MPa/30°C, 50 ppb), 62.11% (300 MPa/50°C/6 pulses  $\times$  50 s, 5 ppb) and 45.49% (300 MPa/50°C/2 pulses  $\times$  150 s, 5 ppb). The results demonstrated that HHP treatment was effective in high patulin concentrations, while p-HHP was effective in low patulin concentrations in clear apple juice. When all pressure applications were compared, the difference was statistically significant ( $p < 0.05$ ) at 5 ppb initial patulin concentration. Similar findings were observed in the literature with citrinin in black table olives.[15] About 2.5 ppb and less citrinin contaminations were degraded more efficiently (56%) at 250 MPa/35°C for 5 min HHP application.

## 4. Conclusions

In this study, the effect of HHP and p-HHP on patulin reduction in commercial clear apple juice was investigated and continuous HHP and p-HHP was compared. The application of pressure to apple juice was found to be effective in reducing the concentrations of patulin. The pressure levels used in this study also guarantee pathogen safety and patulin producer safety. However, the most appropriate pressure and temperature combination for patulin reduction was not determined. Results were variable. The effect of pressure varied depending on the concentration of



patulin in clear apple juice. HHP application for patulin reduction was more effective in high concentrations; however, p-HHP application was more effective in low concentrations of patulin.

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## Disclosure statement

No potential conflict of interest was reported by the authors.

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