

## How sulphur dioxide and storage temperature contribute to patulin degradation in homemade apple juice

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Mycotoxin patulin is one of the quality indicators for apple juice. Like other mycotoxins, it raises consumer health concerns. The issue of low quality is particularly relevant for apples provided by small producers, whose quality control may not be standardised. As sulphur dioxide (SO<sub>2</sub>) is common in fruit preservation against fungi, the aim of this study was to determine how efficient it is in degrading patulin in apple juices stored in real-life conditions. This included refrigerated (4 °C) and non-refrigerated warehouses/environments (30 °C) over 8, 10, 12, and 20 weeks of storage. Apple juice was diluted to 0.010 µg g<sup>-1</sup>, 0.050 µg g<sup>-1</sup>, and 0.100 µg g<sup>-1</sup> of patulin. SO<sub>2</sub> was added to each sample in the amounts of 250 µg mL<sup>-1</sup> and 50 µg mL<sup>-1</sup>. Untreated juice samples for each patulin concentration served as controls under the same experimental conditions. Patulin content was determined with high performance liquid chromatography. The best degradation was observed with 250 µg mL<sup>-1</sup> of SO<sub>2</sub> at 30 °C regardless of the patulin baseline concentration. Although treatment with SO<sub>2</sub> and refrigeration did not fully remove patulin, it was highly efficient over twelve weeks of storage. Our results suggest that patulin levels can be reduced between 33 and 100 % at 30 °C and up to 100 % at 4 °C.

**KEY WORDS:** *contamination; fruit juice; high performance liquid chromatography; mycotoxin; Penicillium expansum*

Patulin is a mycotoxin produced by the *Penicillium*, *Aspergillus*, and *Byssoschlamys* fungal species. It is found in various mouldy fruits, grains, and foods, but is most often associated with contamination of apples and their products (1). The growing apple and apple juice production calls for better quality control, especially with regard to toxic substances like patulin. Particularly vulnerable in terms of health risk are children due to their lower body weight and higher consumption. A recent study has suggested that contamination with patulin could be prevented by removing apple parts showing visible fungal decay (2) or reduced by processing, but its incidence in food products throughout the world witnesses to its stability and resistance to the production methods currently in use (3, 4).

Patulin was first studied as a potential new antibiotic, but research soon demonstrated its adverse effects (5). In rodents, the oral LD<sub>50</sub> of patulin is 29-50 µg g<sup>-1</sup> of body weight. It is reported to be acutely toxic to mice, teratogenic to chicken embryos, and immunosuppressive in mice and rabbits. Furthermore, it is able to inactivate enzymes, mainly as a consequence of its strong affinity for sulphhydryl groups (6). Due to the evidence of its toxicity, the *Codex Alimentarius* and the US Food and Drug Administration (FDA) have recommended a maximum level of 0.05 µg mL<sup>-1</sup> for fruit juices and their products (7, 8). The European

Commission (EC) regulation 1881/2006 (9), in turn, set the recommended limit to 0.025 µg mL<sup>-1</sup> for solid apple products and 0.010 µg mL<sup>-1</sup> for juices and foods for infants.

Recent reports speak of a number of natural and/or synthetic compounds that inhibit mycotoxin growth and production in a variety of commodities (10-13). Some of them focused specifically on the reduction of apple rot and patulin production (14-17).

Sulphur dioxide (SO<sub>2</sub>) is a common preservative used in food industry because of its antifungal activity. In fact, it strongly inhibits mycelium growth and patulin production (3, 10, 12, 18-20). This suggests that it could be used to make up for poor quality control and storage conditions.

The aim of this study was to evaluate its efficiency as a preservative in apple juices stored at different temperatures and storage times that correspond to real-life conditions. The HPLC method for quantitative determination of patulin in samples was developed and validated in terms of linearity, selectivity, trueness, precision, limit of detection, limit of quantification, and ruggedness.

### MATERIALS AND METHODS

#### *Apples*

We used damaged and partly rotten apples of an old Croatian variety collected at a local family orchard.

### Juice preparation

The juice was obtained by crushing 5 kg of unwashed apples in a hydraulic kitchen press.

### Chemicals

High-performance liquid chromatography (HPLC) grade acetonitrile (CAS 75-05-8) was obtained from J. T. Baker (Arnhem, the Netherlands). Perchloric acid (CAS 7601-90-3), diethyl ether (60-29-7), acetic acid (CAS 64-19-7), ethyl acetate (CAS 141-78-6), and sodium bicarbonate (CAS 144-55-8) were obtained from Kemika (Zagreb, Croatia). The patulin (CAS 149-29-1) standard was obtained from Sigma (Steinheim, Germany). The water used for all solutions and HPLC analysis was purified with the Milli-Q system (Merck Millipore, Burlington, MA, USA). Pectinase (EC 3.1.1.11) was obtained from Polyintell (Val-de-Reuil, France).

### Sample preparation and HPLC analysis

All experiments were performed with homemade apple fruit juice without additives. Patulin concentrations were determined with HPLC. The initial concentration measured in juice was  $0.251 \mu\text{g g}^{-1}$ , which was then diluted with 2 % acetic acid to get the baseline patulin concentrations of  $0.100 \mu\text{g g}^{-1}$ ,  $0.050 \mu\text{g g}^{-1}$ , and  $0.010 \mu\text{g g}^{-1}$ . Each solution was made in triplicate. To prevent exposure to light we used tinted flasks.

The first flask was spiked with  $250 \mu\text{g mL}^{-1}$  of  $\text{SO}_2$ , the second with  $50 \mu\text{g mL}^{-1}$ , and the third was not spiked (control). Half of the prepared solutions were kept in a refrigerator ( $4^\circ\text{C}$ ) and the other half in the thermostat set at  $30^\circ\text{C}$  for five months. Patulin was analysed after eight, ten, twelve, and twenty weeks.

The samples for patulin HPLC analysis were prepared in SupelMIP® solid phase extraction (SPE) tubes (Supelco, Bellefonte, USA) using molecularly imprinted polymers to create a three-dimensional network that recognises the shape and functional group positions of a template molecule.

The apple juice was first subjected to the activity of the enzyme pectinase (Polyintell) to become clear.

For analysis we took portions of 2.5 mL, weighed them, diluted with 2.5 mL of 2 % acetic acid, and mixed. The obtained solution was loaded on the SPE column by gravity. To condition the column, 2 mL of acetonitrile were passed through the column, and then 1 mL of water and 1 mL of the diluted sample were added. The column was washed with 1 mL of 1 % sodium bicarbonate and 2 mL of water, and after drying the column under vacuum, 500  $\mu\text{L}$  of diethyl ether was passed through it. Patulin was eluted with 2 mL of ethyl acetate, and one drop of pure acetic acid was added to the eluent to prevent patulin degradation. The eluent was evaporated to dryness under nitrogen. Immediately after drying, we added 500  $\mu\text{L}$  of 0.1 % acetic acid.

For analysis we used the 1200 HPLC system (Agilent, Santa Clara, CA, USA) with the following setup: Synergi Hydro-RP column, 4.6 x 150 mm, 4  $\mu\text{m}$  (Phenomenex, Torrance, CA, USA); 5 % acetonitrile and 0.19 % perchloric acid for mobile phase; 0.2  $\text{mL min}^{-1}$  flow rate. The detection wavelength was set at 276 nm and the sample injection volume was 50  $\mu\text{L}$ . Total run time was 20 min.

### Validation of the applied method

The method was validated for its linearity, selectivity, trueness, precision, limit of detection (LOD), limit of quantification (LOQ), and ruggedness. Linearity was tested in the range of 0.01-0.132  $\mu\text{g g}^{-1}$ . The calibration curve was plotted at eight levels. The trueness of the method was investigated based on the mean recoveries of three spiked sample replicates at each level. Precision, estimated under repeatability conditions and within the reproducibility range, was expressed as a relative standard deviation of the spiked replicates at each of the studied levels. Selectivity was determined based on the profile of chromatograms obtained for standard patulin solutions, patulin-free, and patulin-spiked juice. The ruggedness of the method was tested by registering small changes within the extraction times (0.5 min and 5 min) and evaporation times (5 min and 15 min). The method was verified by inter-laboratory comparison with an accredited laboratory (FAPAS, Sand Hutton, UK; z-score  $\leq 2$ ).

## RESULTS AND DISCUSSION

The LOD and LOQ, established using standard solutions, were  $0.0005 \mu\text{g g}^{-1}$  and  $0.001 \mu\text{g g}^{-1}$ , respectively. The results given in Table 1 prove that the method is satisfactory for the determination of patulin in apple juices. The validation procedure resulted in acceptable mean recoveries of 94-100 %, as well as in acceptable mean intermediate precision of 5.12 %. The same goes for the variation coefficients ( $\text{RSD} < 10\%$ ). Our study has shown that the time needed to reduce patulin depends on storage temperature, initial patulin concentration, and the concentration of  $\text{SO}_2$ .

**Table 1** The results of the patulin analysis method validation

Parameter	Obtained value		
Linearity	k=0.9999		
Trueness	94–100 %		
Precision	RSD	5.12 %	
LOD	0.0005 $\mu\text{g g}^{-1}$		
LOQ	0.0010 $\mu\text{g g}^{-1}$		
Selectivity	Satisfactory		
Ruggedness (extraction time)	0.5 min	RSD	4.90 %
	5.0 min	RSD	4.99 %
(evaporation time)	5.0 min	RSD	4.98 %
	15.0 min	RSD	32.5 %

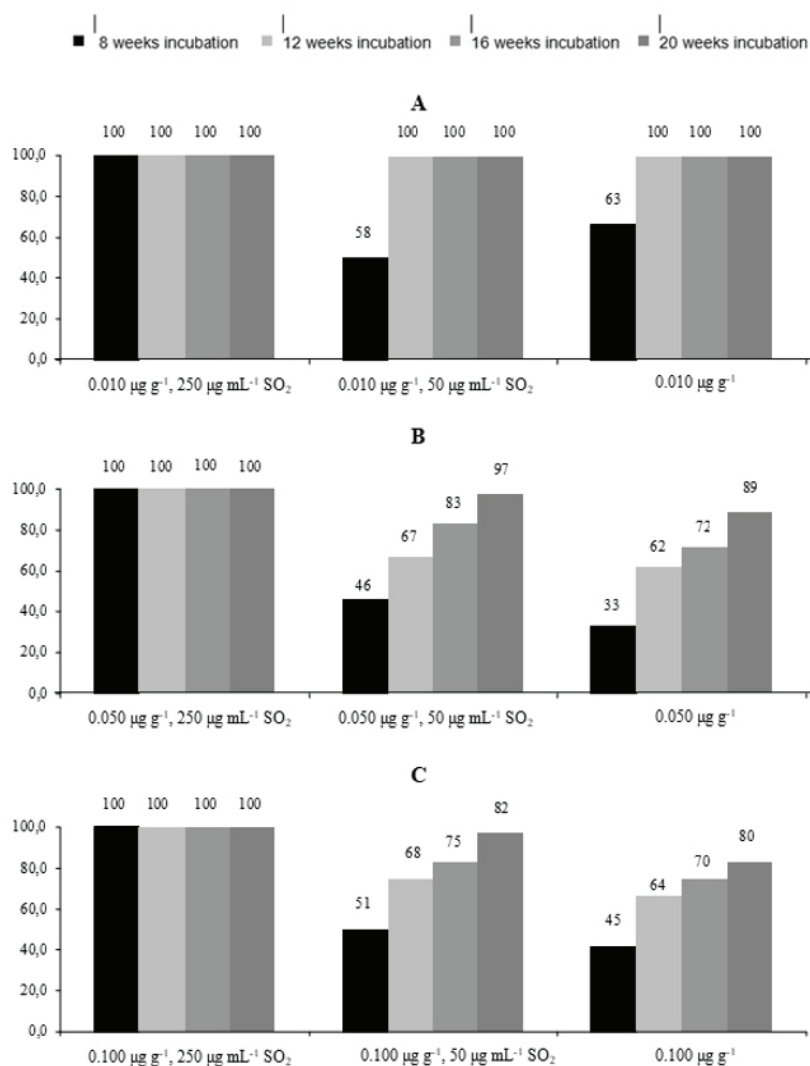
Figure 1 shows the effect on patulin reduction in apple juice over 20 weeks of incubation at 30 °C. The strongest effect (reduction to below LOQ of 0.001 µg g<sup>-1</sup>) was observed for the treatment with the highest SO<sub>2</sub> concentration regardless of the baseline patulin concentration, which supports the findings of Tabatabaie et al. (3) that higher temperatures favour patulin degradation. A similar reduction of the *Byssochlamys nive* biomass was reported for the combination of SO<sub>2</sub> and temperature of 24, 30, and 37 °C by Ough and Were (21). Temperature treatments, however, can diminish clarity and organoleptic properties of such juices (3). Koca et al. (22) also reported a 78-100 % reduction of patulin in samples treated with ascorbic acid alone (without SO<sub>2</sub>) and stored at 30 °C, but note that even our control samples showed complete degradation with lowest baseline patulin concentrations.

However, untreated patulin at the highest baseline concentrations of 0.100 µg g<sup>-1</sup> did not drop below the limit for safe consumption recommended by the EC (9) for fruit juices (0.050 µg g<sup>-1</sup>) in the first eight weeks of storage,

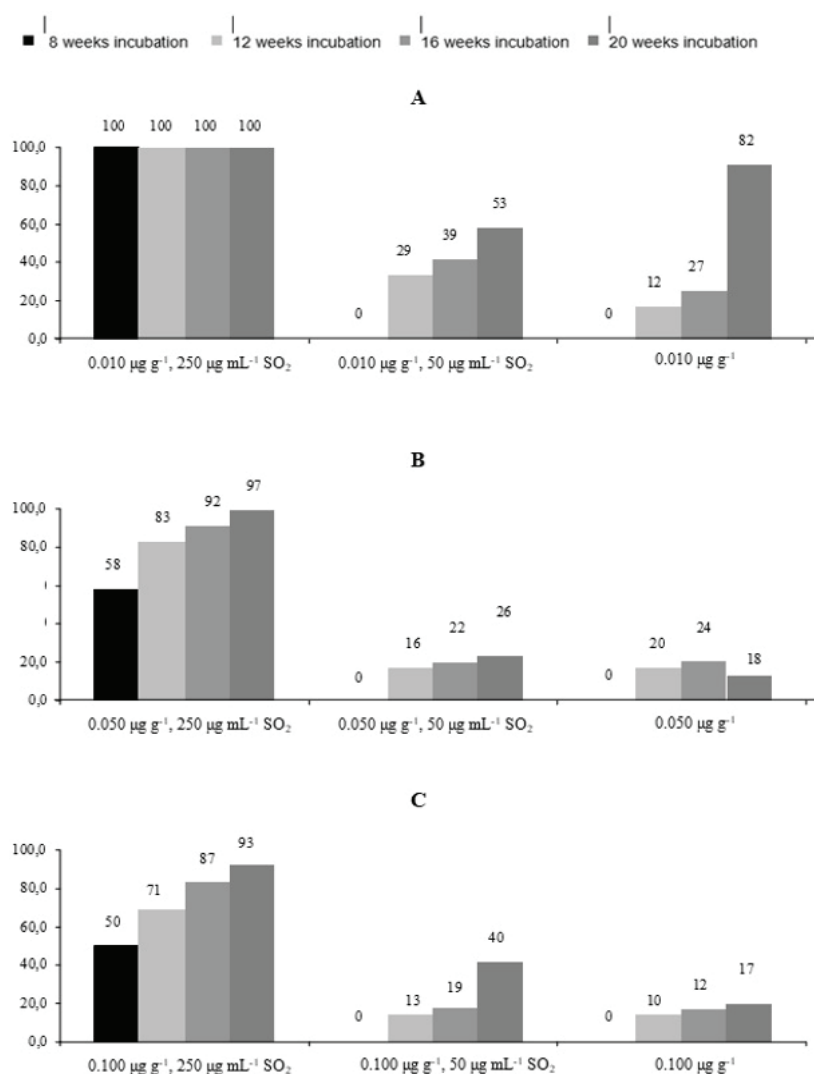
which means that contamination with higher patulin levels requires treatment with SO<sub>2</sub> at higher temperatures (e.g. 30 °C in our study) with incubation of over eight weeks. With the exception of the above mentioned case, no significant differences were observed between control (baseline patulin concentrations of 0.050 µg g<sup>-1</sup> and 0.100 µg g<sup>-1</sup>) and samples treated with 50 µg mL<sup>-1</sup> SO<sub>2</sub>. Here patulin degradation for both experimental concentrations during 20 weeks was almost similar.

Figure 2 shows patulin reduction in samples refrigerated at 4 °C. Compared to storage at 30 °C (Figure 1), it is noticeably less effective, except for the baseline patulin concentration of 0.010 µg g<sup>-1</sup> treated with the highest SO<sub>2</sub> concentration of 250 µg mL<sup>-1</sup>, where patulin levels dropped below the LOQ. However, lower SO<sub>2</sub> concentrations entailed lower patulin degradation.

Patulin has been shown to be unstable in the presence of sulphur-containing compounds (23). A study of Burroughs et al. (24) reported a 90 % of patulin reduction after two days of treatment with a 200 ppm SO<sub>2</sub> solution.



**Figure 1** Percentage of patulin reduction during storage at 30 °C. Baseline patulin concentrations: A - 0.010 µg g<sup>-1</sup>; B - 0.050 µg g<sup>-1</sup>; C - 0.100 µg g<sup>-1</sup>



**Figure 2** Percentage of patulin reduction during storage at 4 °C. Baseline patulin concentrations: A - 0.010 µg g<sup>-1</sup>; B - 0.050 µg g<sup>-1</sup>; C - 0.100 µg g<sup>-1</sup>

Cavallito et al. (25) later reported that only 100 ppm of sulphur dioxide reduced patulin content by 50 % in 15 min. The differences between these reports and our findings could be attributed to differences in the composition of the sample solutions, as interfering components may have been present. Patulin is also believed to react with a number of other sulphur-containing compounds such as cysteine, *N*-acetylcysteine, and glutathione (12) because it has a strong affinity for binding covalently to sulfhydryl groups as well as to amino, thiol, and NH<sub>2</sub> groups (26). While the resulting adducts are still toxic, their toxicity is 100 times lower than that of patulin (27, 28).

In summary, among the 36 apple juice samples with six patulin and SO<sub>2</sub> combinations, 70 % showed reduction to levels below the EC safety limit (9). This suggests that adjusting SO<sub>2</sub> and storage temperatures is a promising approach for fruit juice industries to preventing contamination with patulin.

Future studies should include variations in temperature and SO<sub>2</sub> concentrations to determine the lowest conditions

for patulin reduction and their influence on organoleptic properties of the juices.

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### Utjecaj sumpornog dioksida i temperature skladištenja na razgradnju patulina u domaćem jabučnom soku

Mikotoksin patulin jedan je od pokazatelja kakvoće jabučnog soka. Poput drugih mikotoksina, prisutnost patulina predstavlja zdravstveni problem. Pitanje loše kakvoće jabuka odnosi se na mala obiteljska gospodarstva, gdje se kakvoća korištenih jabuka ne može standardizirati. Prisutnost plijesni i mikotoksina zdravstveni je problem, a SO<sub>2</sub> pospješuje razgradnju patulina. Svrha ove studije bila je odrediti razgradnja patulina u jabučnom soku tretiranim sa SO<sub>2</sub> pri različitim temperaturama skladištenja. Utjecaj SO<sub>2</sub> na razgradnju patulina ispitivana je u uzorcima pohranjenima u svakodnevnim uvjetima. To uključuje hladnjak (4 °C) i nehlađena skladišta/okoliš (30 °C) tijekom 8, 10, 12 i 20 tjedana. Jabučni sok razrijeđen je na koncentracije 0,010 µg g<sup>-1</sup>, 0,050 µg g<sup>-1</sup> i 0,100 µg g<sup>-1</sup> patulina. U svaki uzorak dodan je SO<sub>2</sub> u količini od 250 µg mL<sup>-1</sup> i 50 µg mL<sup>-1</sup>. Uzorci soka u koji nije dodan SO<sub>2</sub> za svaku koncentracijsku razinu patulina poslužili su kao kontrola i bili su pod istim eksperimentalnim uvjetima. Patulin u uzorcima određivan je pomoću tekućinskog kromatografa visoke djelotvornosti (HPLC). Najveća razgradnja patulina zabilježena je u termostatskim uvjetima s koncentracijom SO<sub>2</sub> od 250 µg mL<sup>-1</sup>, bez obzira na koncentraciju patulina kontrolnog uzorka. Iako hlađeni uvjeti i dodatak SO<sub>2</sub> nisu potpuno uklonili patulin iz uzoraka, nakon dvanaest tjedana skladištenja uočena je visoka učinkovitost razgradnje. Rezultati pokazuju da se razine patulina mogu smanjiti 33-100 % pri temperaturi od 30 °C i 0-100 % pri 4 °C.

**KLJUČNE RIJEČI:** *kontaminacija; voćni sok; tekućinska kromatografija visoke djelotvornosti; mikotoksin; Penicillium expansum*