Reduction of Patulin during Apple Juice Clarification

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ABSTRACT

Patulin is a mycotoxin produced by a number of molds involved in fruit spoilage. This compound is carcinogenic and teratogenic. Various methods are currently used to reduce the levels of patulin in apple juice, namely, charcoal treatment, chemical preservation (sulfur dioxide), gamma irradiation, fermentation, and trimming of fungus-infected apples. Many of these processes are expensive and time-consuming. Therefore, there is a need to find a convenient and economical process to control patulin levels. This study was undertaken to evaluate the effectiveness of several clarification processes for the reduction of patulin. Clarification was carried out on a laboratory scale. Apple pulp was spiked with patulin, pressed, and clarified using four different processes, namely, fining with bentonite, enzyme (pectinase) treatment, paper filtration, and centrifugation. Patulin was recovered from the clarified juice by liquid-liquid extraction, and solid-phase chromatography was used for sample cleanup prior to analysis by high-performance liquid chromatography (HPLC). The minimum detectable limit using HPLC was 20 μg/liter. Pressing followed by centrifugation resulted in an average toxin reduction of 89%. Total toxin reduction using filtration, enzyme treatment, and fining were 70, 73, and 77%, respectively. Patulin reduction was due to the binding of the toxin to solid substrates that was verified by analyzing the clarified juice as well as the filter cake, pellet, and sediment. The combined concentrations correlated to the spiked concentration. These results reveal that clarification was successful in the reduction of patulin levels in apple juice. However, clarification resulted in high levels of patulin in the pressed pulp after filtration and centrifugation, and this could be harmful if they are used as animal feeds.

Patulin (4-hydroxy-4H-furo[3,2-c]pyran2[6H]-one), a mycotoxin, is a secondary metabolite produced by several fungi, primarily Aspergillus and Penicillium species. It was isolated by Birkinshaw et al. (1) from Penicillium patulum and Penicillium expansum. P. expansum is a well-known postharvest pathogen that causes blue mold rot in apples and also produces the mycotoxin patulin (25). Patulin-producing fungi occur in a variety of foods, but only apple juice and apple cider have been found to be contaminated naturally with patulin (26).

The toxicological properties of patulin have recently been reviewed, and patulin has been reported to be mutagenic and to cause neurotoxic, immunotoxic, genotoxic, and gastrointestinal effects in rodents, although little scientific evidence exists to support media contention that patulin is carcinogenic to humans (12). The international maximum permitted limit for patulin contamination is 50 ppb (10). A survey carried out in South Africa for the presence of patulin in apple products from 1996 to 1998 revealed that 5 out of 22 apple juice samples (23%) analyzed contained patulin levels between 10 and 45 ppb. Patulin was also found in 29% of infant apple products surveyed, with toxin concentration ranging between 5 and 20 ppb (3). Patulin was detected in all 215 apple juice concentrates tested from three different suppliers at concentrations ranging from 7 to 375 ppb and 43% of the samples exceeded the patulin contamination level of 50 ppb (10).

Raw apple juice is normally clarified to remove suspended solid material (19). This can be accomplished in one of two ways: enzymatically and nonenzymatically. The use of pectinase enzymes breaks down the pectin coat surrounding protein particulates in the emulsion, allowing the particles to aggregate and sediment (17). Nonenzyme clarification involves breaking the emulsion by other means, the most common of which is heat treatment. Other techniques include addition of bentonite, gelatin, casein, tannic acid-protein combinations (13), and chitosan (22). Mechanical separation techniques like decanting, centrifugation, filtration (27), pressure filtration, and rotary vacuum filtration and more recently membrane (ultrafiltration) filtration (16, 21) are also used to separate the solid matter from the juice in the manufacture of apple juice (17, 19).

A study on the effect of down-line processing has emphasized the need to reduce the levels of patulin entering the factory, as subsequent processes, other than the addition of activated carbon, have little or no influence on the levels of patulin in the final product (4). The use of activated charcoal (while an effective agent for the reduction of patulin levels) is not feasible to apply on a commercial scale (4). Alternative methods need to be found to control patulin during down-line processing.

This paper describes the use of several conventional techniques (fining with bentonite, paper filtration, pectinase treatment, and centrifugation) to evaluate the removal of patulin during the clarification processes involved in apple juice manufacture.

MATERIALS AND METHODS

Standards. Golden Delicious apples were bought from local supermarkets in the Durban area. Apple juice clarification was...
carried out on a laboratory scale. Patulin was purchased from Sigma Chemical Company (Atlasville, South Africa). Stock solutions of 100 and 1,000 μg/ml were prepared in chloroform, dried, and stored at −20°C. Working standards of 0.1, 1.0, and 10 μg/ml patulin were prepared by dilution of stock solutions with water:acetonitrile (9:1).

**Apple juice clarification.** Approximately 1 kg of apples was sliced, blanched (60 to 70°C), and homogenized using a commercial blender (Waring Products Division, New Hartford, Conn.). The crushed apple pulp was spiked with patulin to yield a concentration of 2 μg/g. The pulp was pressed using a double layer of cheesecloth and the juice collected by vacuum extraction. In order to monitor the total dissolved solids in the juice from different pressings, the Brix was determined by the method of Zubrick.

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**Combination treatments.** Four combination treatments, viz., fining and centrifugation, enzyme treatment and centrifugation, filtration and fining, and filtration and enzyme treatment were tested by performing the first treatment followed by the second as described above.

**Patulin purification.** Samples were prepared for analysis by the method of Rovira et al. (20) with the following changes: 50 ml of clarified apple juice was extracted three times with equal volumes of ethyl acetate. The ethyl acetate fractions were combined and evaporated to dryness under reduced pressure over approximately 1 g anhydrous sodium sulfate. The dried residue was redissolved in 20 ml chloroform and dried for 1 h. The chloroform solution (5 ml) was introduced into a Sep-Pak SiO$_2$ cartridge (Waters Chromatography Division, Millipore Corp., Milford, Conn.), previously conditioned with 5 ml chloroform. The cartridge was washed with 1 ml chloroform, 1 ml chloroform:ethyl acetate (4:1), and 1 ml chloroform:methyl acetate (1:1). Two milliliters of chloroform:ethyl acetate (1:4) was used to elute patulin. The eluant was evaporated under a gentle stream of nitrogen, and the dried residue was dissolved in 1 ml water:acetonitrile (9:1).

**Thin-layer chromatographic analysis.** The method of Prieta et al. (18) was used. Ten microliters of the sample and patulin standards were spotted on thin-layer chromatography plates (silica gel 60 F$_34$4, Merck) and developed in a mobile phase consisting of toluene:ethyl acetate:90% formic acid (5:4:1). The plates were sprayed with 0.5% 3-methyl-2-benzothiazolinone hydroxy- droadilride hydrate, heated in an oven at 130°C for 15 min, and examined under long-wave UV light. Patulin appeared as a yellow-brown spot with an $R_f$ value of 0.5. Once the toxin was detected, their levels were quantified by HPLC analysis.

**HPLC analysis.** A Merck-Hitachi model D-7000 HPLC system equipped with an L-7400 variable-wavelength UV detector (Merck-Hitachi, Tokyo, Japan) set at 276 nm, an L-7200 autosampler (Merck-Hitachi), and an L-7100 pump (Merck-Hitachi) was used. The mobile phase was water:acetonitrile (99:1) at a flow rate of 1 ml/min. Twenty-microliter injections were separated in a Lichrosphere 100 RP-18 analytical column (250 nm by 4 mm) at room temperature. A standard curve was constructed for patulin concentration (concentration [μg/ml] of patulin versus peak area). Patulin concentration of samples was calculated with the following equation:

$$\text{patulin (μg/liter) = } \left( \frac{C}{V_i} \right) \times \left( \frac{V_f}{V_i} \times 1,000 \right)$$

where $C$ is the concentration of patulin in μg/ml in the final solution, $V_i$ is the total volume of the final solution (ml), and $V_f$ is the initial volume (ml) of the apple juice taken for extraction. All experiments were performed in triplicate, and mean and standard deviation of HPLC results for patulin recovery were calculated.

**RESULTS AND DISCUSSION**

Clarification was carried out on freshly prepared pulp that was spiked with patulin to yield a final concentration of 2 μg/ml. Initial HPLC data were erratic due to the presence of a coextract (probably 5-hydroxymethylfurfuryl [HMF]) that peaked at a similar retention time to patulin. HMF is formed as a result of dehydration of ketopentoses, particularly in acidic or high temperature environments (14). The pulp pH ranged between 3.3 and 3.7 in our study. This may have contributed to the formation of HMF.

Spa- nos et al. (23) reported increasing levels of HMF in apple juice during storage.

Forbito and Babsky (9), who used the same mobile phase for HPLC analyses as that used initially in our study, also observed the presence of an unknown peak overlapping with that corresponding to patulin. However, Chudziak and Trojanowicz (6), Brause et al. (2), and Gokmen and Acar (11) were able to obtain effective separation of these two peaks (HMF and patulin) by reversed-phase liquid chromatography and the use of an alternative mobile phase, viz., water:acetonitrile (1:99). In our study, this mobile phase did improve separation of the peaks significantly but not totally. Complete separation was achieved with the use of the drying agent, anhydrous sodium sulfate. Brause et al. (2) reported that patulin may be destroyed when a “wet” ethyl acetate extract is evaporated to dryness. The method initially used in this study required the ethyl acetate to be
dried completely immediately after extraction from the aqueous sample without the use of a drying agent. It is possible that patulin may have degraded during the drying step and that the breakdown products in combination with HMF caused peak interference. Further studies will be required to determine the actual breakdown products and their chemical properties. The addition of anhydrous sodium sulfate in the first drying step during sample preparation improved toxin recovery and analysis because toxin and coextract peaks were completely resolved.

The limit of detection for patulin in this study was 20 μg/liter. The average patulin recovery rate from spiked apple juice was 84% for the three patulin concentrations tested (results not shown). The 16% loss of patulin may be due to patulin damage or destruction during the drying stage of the extraction as well as patulin loss in the cartridges. Pressing of the apple pulp to extract the juice was done prior to testing the clarification procedures. The results obtained from the single-clarification processes are summarized in Table 1. An aliquot of spiked (2 μg/ml) pulp (unprocessed) was used as a control in each experiment. Although the pulp was not processed, a loss of patulin of 19% was observed. This value reflects the recovery potential of the extraction and clean-up method from apple juice. Most of the patulin was removed during the pressing step. The apple pulp had been spiked with 2 μg/ml patulin, and approximately 0.95 μg/ml was recovered after the pressing step resulting in an average loss of 52.5%. After pressing, the juice was subjected to individual clarification processes, viz., filtration, fining, centrifugation, and enzyme treatment. Centrifugation was the most effective clarification procedure with an average patulin removal of 89%. This value is not the true reduction potential of this treatment only because it includes the patulin already removed in the pressing step as well as the patulin levels lost in the extraction and clean-up steps. The patulin reduction achieved by centrifugation alone is therefore 20.5%. Fining was the second-most effective clarification process, and it reduced the toxin level by 8.5%. Filtration and enzyme treatment were less effective than the two treatments already described. No patulin reduction occurred with filtration and only 4.5% with enzyme treatment. Bullerman (5) reported that patulin may bind to solid material. The effectiveness of these clarification treatments in toxin removal can be attributed to the adherence of patulin to solid particles because the patulin loss in the juice can be recovered in the centrifugation pellet and sediment from the fining process. The combined concentrations correlated with the spiked concentration of 2 μg/ml.

Four combination treatments, viz., centrifugation and fining, centrifugation and enzyme treatment, fining and filtration, and filtration and enzyme treatment were tested (Table 2). Pressing resulted in a 48% loss of patulin. Surprisingly, the combination treatments resulted in a lower percentage loss of patulin than the individual treatments. Centrifugation and fining produced the greatest loss of patulin (20.5%), which was the value obtained for centrifugation alone, followed by centrifugation and enzyme treatment (17%), fining and filtration (12%), and filtration and enzyme treatment (7.5%). The lower than expected values obtained for the combined treatments can be explained by the fact that patulin removal is due to the binding of the toxin to particulate matter that is removed from the juice. During centrifugation, patulin bound to particulate matter will be effectively removed. This study has shown that most of the toxin removed is located in the press cake and the pellet formed after centrifugation. However, when centrifugation is combined with enzyme treatment, in this case pectinase, pectin particles present in the juice would be degraded by the enzyme and centrifugation will no longer be as effective.

### Table 1. Evaluation of single clarification treatments in patulin removal

<table>
<thead>
<tr>
<th>Process</th>
<th>Patulin concentration (μg/ml)</th>
<th>% loss (total)</th>
<th>% loss (minus pressing)</th>
<th>% loss (minus recovery)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pressing</td>
<td>0.95 ± 0.03</td>
<td>1.05</td>
<td>52.5</td>
<td>—</td>
</tr>
<tr>
<td>Centrifugation</td>
<td>0.22 ± 0.02</td>
<td>1.78</td>
<td>89</td>
<td>36.5</td>
</tr>
<tr>
<td>Fining</td>
<td>0.46 ± 0.05</td>
<td>1.54</td>
<td>77</td>
<td>24.5</td>
</tr>
<tr>
<td>Enzyme treatment</td>
<td>0.55 ± 0.05</td>
<td>1.45</td>
<td>73</td>
<td>20.5</td>
</tr>
<tr>
<td>Filtration</td>
<td>0.68 ± 0.08</td>
<td>1.32</td>
<td>70</td>
<td>17.5</td>
</tr>
<tr>
<td>Control</td>
<td>1.62 ± 0.08</td>
<td>0.38</td>
<td>19</td>
<td>—</td>
</tr>
</tbody>
</table>

### Table 2. Evaluation of combination clarification treatments in patulin removal

<table>
<thead>
<tr>
<th>Process</th>
<th>Patulin concentration (μg/ml)</th>
<th>% loss (total)</th>
<th>% loss (minus pressing)</th>
<th>% loss (minus recovery)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pressing</td>
<td>1.03 ± 0.06</td>
<td>0.97</td>
<td>48.5</td>
<td>—</td>
</tr>
<tr>
<td>Centrifugation and fining</td>
<td>0.34 ± 0.04</td>
<td>1.66</td>
<td>83</td>
<td>34.5</td>
</tr>
<tr>
<td>Centrifugation and enzyme treatment</td>
<td>0.41 ± 0.04</td>
<td>1.59</td>
<td>79.5</td>
<td>31</td>
</tr>
<tr>
<td>Fining and filtration</td>
<td>0.51 ± 0.03</td>
<td>1.49</td>
<td>74.5</td>
<td>26</td>
</tr>
<tr>
<td>Filtration and enzyme treatment</td>
<td>0.60 ± 0.05</td>
<td>1.4</td>
<td>70</td>
<td>21.5</td>
</tr>
<tr>
<td>Control</td>
<td>1.66 ± 0.08</td>
<td>0.34</td>
<td>17</td>
<td>—</td>
</tr>
</tbody>
</table>
Patulin will be bound to smaller particles or remain unbound if there is no particulate matter available and would therefore not sediment during centrifugation. The combination of fining with centrifugation also resulted in lower toxin reduction than centrifugation alone. This could be due to loss of particulate matter during the fining step. However, this treatment is more effective than the fining step alone.

Our results indicate that centrifugation is an effective clarification process for the reduction of patulin. It also has the potential to be implemented on an industrial scale because most juice-making plants have a pre-existing centrifugation step. The centrifugal forces employed may have to be modified in order to remove as much of the particulate matter as possible so as to obtain efficient reduction in patulin levels. Patulin losses obtained in our study (89% for centrifugation and 77% for fining) are comparable to those obtained with activated charcoal treatment (45 to 64%) that has the disadvantage of being an expensive and time-consuming treatment (4). Trimming (15) and the use of insecticides (7) result in 93 to 99% and 85 to 100% loss of patulin, respectively. However, the first method is time consuming and labor intensive and the second has safety implications regarding the use of organophosphates. Alcoholic fermentation (24) and ammoniation (8) produced >99% and 99.8% patulin loss but the former can only be used for the production of cider and not juices and in the case of the latter the sample was not fit for human or animal consumption and had to be discarded. The clarification procedures tested during this study are simple to implement, cost effective, neither time consuming nor labor intensive. The only concern is for industries that use apple juice by-products like the filter and cake press. These products have the potential of containing high levels of patulin, and caution will have to be practiced to avoid health hazards to humans and animals.

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REFERENCES