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Occurrence of patulin in the commercial processing of apple juice

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Patulin, a mutagenic and genotoxic secondary metabolite, is produced in apples by several fungi, of which *Penicillium expansum* Link is the most significant. The importance of removing contaminated apples from the initial processing line during apple juice production was studied during three consecutive seasons. Mean patulin concentration in non-processed apples over the three seasons was 2010 ± 949 ng g^{-1}. The decrease in patulin concentration to 440 ± 253 ng g^{-1} after an initial water wash step was followed by further reduction to 200 ± 183 ng g^{-1} ($P < 0.05$) after the removal of rotten and damaged apples. A further study was undertaken to determine the effects of processing on patulin concentrations in apple juice. Composite samples were independently withdrawn at four sequential stages during manufacture, namely, pre-concentration, depectinization/charcoal/ultra-filtration, passage through a resin-based absorber, and final concentration. During the pre-concentration stage (pasteurization), a statistically significant ($P < 0.0001$) decrease in the mean *P. expansum* count from 4.07 to 0.79 $\log_{10}$ colony forming units (cfu) ml^{-1} accompanied a significant increase ($P < 0.05$) in patulin concentration from 105 ± 44 ng ml^{-1} to 165 ± 49 ng ml^{-1}. These results were probably due to the higher temperature and total solids concentration (Brix), respectively, during pasteurization. The combined depectinization/charcoal/ultra-filtration stage yielded a significant decrease in mean patulin concentration from 110 ± 35 ng ml^{-1} to 75 ± 18 ng ml^{-1} ($P < 0.05$), which was probably due to the adsorption of patulin on the activated charcoal. No further removal of patulin occurred during the remainder of the juicing process.

Introduction

Patulin is a toxic secondary metabolite produced by several fungal species of the genera *Penicillium* and *Aspergillus*, of which *Penicillium expansum* Link is the most common.[1] Contamination of apples by *P. expansum*, referred to as 'blue mould rot', is normally associated with damaged fruit.[2,3] Patulin exhibits mutagenic properties and causes neurotoxic, immunotoxic, genotoxic and gastrointestinal effects in rodents.[4]

International concern for the occurrence of significant residual patulin concentrations in apple products was emphasized by a series of press releases in the United Kingdom following a survey conducted by the Ministry of Agriculture, Fisheries and Food.[5] Several countries and the International Fruit Juice Association have recommended residual patulin concentrations of less than 50 parts per billion (ppb) for apple products intended for human consumption.[6] Residual patulin concentrations in apple juice products may be indicative of the quality of apples used for their production.[7] Recommendations have been made by the British Soft Drinks Association (BSDA) to minimize patulin in apple juice.[8] These recommendations include measures relating to horticultural practices, harvesting, post-harvest transportation, storage, processing, packaging and bulk transportation. The formation of blue mould rot in apples may be associated with particular fungal species, cultivars and environmental conditions.[1,9] The removal of apples contaminated with fungi before processing reduces patulin concentrations significantly in apple juice products.[10–12] Several processing procedures involving charcoal treatment, addition of ascorbic acid, irradiation and alcoholic fermentation have been reported to further reduce patulin concentrations in apple juice.[13–16]

Owing to restricted processing capacities and limited cold storage facilities at a South African factory study site, apples are frequently stored in the open (deck storage) over extended periods, increasing their susceptibility to fungal rot and hence patulin contamination. The initial factory processing consisted of a water wash and subsequent manual sorting to remove rotten fruit. The influence of these steps on patulin concentrations in overripe Granny Smith apples entering the extraction process was studied over three consecutive seasons. The results of the first and second seasons of this project have been published by Sydenham *et al.*[12,13] This paper reports the results of the third season. In addition, apple juice processing was studied to determine the influence of various operations on patulin concentration and fungal contamination in apple juice as it proceeds through the factory. Commercial processing consists of an initial pre-concentration and pasteurization process in which juice is rapidly heated to approximately 55°C and the concentration rose from approximately 10° to 14° Brix. Subsequently, the juice was subjected to enzymatic depectinization, including simultaneous charcoal treatment, followed by ultrafiltration. The ultrafiltrate was passed through a resin absorption system before the final concentration of the juice to approximately 70° Brix.

Materials and methods

Initial processing of apples. During 1996, a consignment of Granny Smith apples stored in the open (deck storage) for approximately 35 days after harvesting was sampled at four stages during initial processing. These four stages were: (A) apple crates entered the factory from deck storage; (B) after a water wash treatment; (C) after both wash and hand sorting to remove rotten fruit; and (D) the rotten fraction removed by hand sorting. The sampling procedure, which was performed after 0, 2.5 and 5 h, was the same as used in similar studies during the two previous seasons.[12,13] At point A (deck storage), three bins of apples, each containing approximately 360 kg and representative of the apples entering the factory, were sampled by an automatic random sampler which collected 25 kg laboratory samples representative of each bin. Over a corresponding 30-minute period, apples were randomly selected by hand (to fill separate 360 kg bins) from stages B and C. Laboratory samples of 25 kg were withdrawn from these individual bins using the same random subsampling method as for the apples at stage A. Additionally, a 25 kg laboratory sample of the hand-sorted, visibly rotten and damaged apples removed from the sorting table was collected as representative of the rotten fraction (stage D). Apples from each 25 kg laboratory sample were individually sliced, the juice was extracted, mixed for homogeneity and aliquots were stored at –4°C for analysis.

Apple juice processing. For ease of sampling, this study was conducted in three independent parts, namely, (1) pre-concentration, (2) depectinization including charcoal treatment and ultrafiltration, and (3) concentration. Ten composite apple juice

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samples were independently withdrawn over 10-minute intervals at each sequential stage during processing, as detailed below.

(1) *Pre-concentration*: samples were collected at the buffer tank into which apple juice, emanating from the juice extractors, was added and mixed. Further samples were collected after the pre-concentration.

(2) *Depectinization*: samples were collected before depectinization. This was the same sampling point as for after pre-concentration, except that this part of the study was conducted independently and at a different time from the pre-concentration stage. Further samples were collected after depectinization, charcoal treatment and ultrafiltration.

(3) *Concentration*: samples were collected from an 80 000-litre clear-juice buffer tank, before the juice entered the absorber. Samples were then collected after the juice had passed through the absorber. Samples were subsequently collected from a small buffer tank after the absorber and before entering the concentration units. Finally, samples were collected after the juice had passed through the concentration units.

The composite samples collected at each of the above sampling points were stored at –4°C for chemical and mycological analyses.

Chemical analyses. Apple juice samples obtained prior to depectinization were incubated with pectinase enzymes for two hours at 58°C to remove pectins. The samples were cooled to room temperature and filtered. Juice from the final concentration stage was adjusted to 12° Brix. The concentration of patulin was determined in accordance with the method described in ref. 8 with minor modifications.

An aliquot (5 ml) of clear or clarified juice was sequentially extracted with two 10-ml portions of ethyl acetate in a separating funnel for 1 minute. The aqueous phase was discarded. The pooled ethyl acetate fractions were washed in the same separating funnel with 2 ml aqueous sodium carbonate solution (1.4% w/v) to remove phenolic acids. This step was completed within 1–2 minutes, since patulin is unstable under alkaline conditions. The ethyl acetate solution was transferred into a 50-ml glass-stoppered extraction tube to which 1 g anhydrous sodium sulfate was added as a drying agent. After immediate swirling, the solution was filtered and transferred to an evaporating flask. The extraction tube was rinsed with two 2-ml portions of ethyl acetate. The organic solvent was removed by rotary evaporation at 40°C. The residue was re-dissolved in 250 μl mobile phase and retained for analysis by high-performance liquid chromatography (HPLC).

Chromatographic analyses. Purified extracts were analysed by reversed-phase HPLC using a 150 × 4.6 mm i.d. stainless steel column packed with Luna C_{18} material of 5 μm diameter particle size (Phenomenex, Torrance, California). The eluate was monitored with a Hewlett-Packard 1040A diode array detector, programmed to record ultraviolet spectra between 210 and 350 nm. The mobile phase ($CH_3CN:H_2O$ – 10:90) was delivered at 1 ml min^{-1} from a Waters Model 590 pump. An authentic patulin standard (Sigma) and the purified extracts were introduced (10–20 μl) via a Rheodyne injector. The concentration of the standard solution was determined by UV spectroscopy at 275 nm, against a solvent blank, using the molar mass and molar extinction value reported by the Association of Official Analytical Chemists.[17]

Mycology. The determination of colony forming units (cfu's) in each apple juice sample was performed for a series of 10-fold dilutions (i.e., $1:10^{-1}$ to $1:10^{-8}$) of non-enzyme-treated juice in sterile water. Aliquots (1 ml) of the respective dilutions were added to sterile Petri dishes containing approximately 20 ml molten malt-extract agar cooled to 45°C. After thorough agitation, the agar was allowed to set and the plates were incubated in the dark at 25°C for seven days. Counts of *P. expansum* were recorded separately as $\log_{10}$ cfu ml^{-1} and were identified according to the methods of Pitt.[18]

Statistics. Statistical analysis was performed on the log-transformed analytical and mycological data using the Systat Software Package (Version 7, SPSS Inc., Chicago). Analysis of variance was performed using general ANOVA, together with the Bonferroni adjustment for pairwise comparisons.

Results and discussion

Initial processing

The mean patulin concentrations and *P. expansum* counts recorded from the studies on the influence of initial processing of apples during three consecutive seasons are presented in Tables 1 and 2, respectively. The wide variation in the individual patulin concentrations, indicated by the standard deviations at the four sampling points, implies a heterogeneous distribution of the metabolite. The results obtained from the 1996 samples confirmed previously published data from the 1994 and 1995 seasons that patulin concentrations and *P. expansum* counts in deck-stored apples were extremely high.[12,13] Contamination progressively declined following the water wash step and the hand sorting in all three study seasons. These reductions, together with the high mean patulin concentrations (3330 ± 2556 ng g^{-1}) recorded in the rotten and damaged fruit, indicate the importance of removing rotten and damaged fruit prior to further processing. Fungal counts showed a numerical reduction from the incoming apples through to the washed and sorted fruit,

Table 1. Mean patulin concentrations in apple juice samples from deck storage and initial processing over three consecutive seasons.

	Patulin concentration (ng g^{-1})			
	A	B	C	D
Season	Deck storage	Wash treatment	Wash and sort	Rotten fraction
1994	920 ± 1028 abc	190 ± 31 b	55 ± 38 c	2335 ± 950 a
1995	2445 ± 1170 a	695 ± 146 a	405 ± 88 a	6235 ± 1030 b
1996	2660 ± 1047 a	430 ± 101 bc*	135 ± 59 c*	1425 ± 795 ab
Mean	2010 ± 949 a	440 ± 253 ab	200 ± 183 b	3330 ± 2556 a

*These samples were washed with hot water in contrast to the 1994 and 1995 seasons.
Values in a row followed by the same letter are not significantly different ($P > 0.05$), while those followed by different letters are significantly different ($P < 0.05$).

Table 2. Mean *Penicillium expansum* counts in apple juice samples from deck storage and initial processing over three consecutive seasons.

	Penicillium expansum counts ($\log_{10}$ cfu ml^{-1})			
	A	B	C	D
Season	Deck storage	Wash treatment	Wash and sort	Rotten fraction
1994	4.49a	3.74a	3.71a	4.01a
1995	5.20a	4.36b	4.01c	6.68d
1996	6.69a	5.46a*	5.38a*	5.82a
Mean	5.46a	4.52a	4.37a	5.50a

*These samples were washed with hot water in contrast to the 1994 and 1995 seasons.
Values in a row followed by the same letter are not significantly different ($P > 0.05$), while those followed by different letters are significantly different ($P < 0.05$).

Table 3. Mean patulin concentrations and *Penicillium expansum* counts in apple juice samples collected during apple juice processing.

	Pre-concentration		Depectinization		Concentration			
	Pre-concentration In	Pre-concentration Out	Depectinization In	Ultra-filtration Out	Absorber In	Absorber Out	Concentrator In	Concentrator Out
Patulin concentration (ng ml^{-1})	105 a	165 b	110 a	75 b	125 b	155 a	120 a	130 a
s.d.	44	49	35	18	41	11	20	10
P. expansum counts (log_{10} cfu ml^{-1})	4.07a	0.79b	1.17a	0.97a	1.09a	0.95a	0.30b	1.35a
Range	3.28–4.90	0–1.60	0–1.70	0–1.48	0–1.85	0–1.60	0–1.00	0–2.04

Means for each pair of columns (i.e. for each In/Out treatment) followed by the same letter are not significantly different ($P > 0.05$), whereas means followed by different letters are significantly different ($P < 0.05$).

although these declines were only statistically significant ($P < 0.05$) in the 1995 samples. Because patulin concentrations after sorting may be higher than the recommended limit of 50 ng g^{-1}, methods for the further reduction of patulin concentrations in apple juice should be considered.

Apple juice processing

A significant ($P < 0.05$) increase in patulin concentration from 105 ± 44 ng ml^{-1} to 165 ± 49 ng ml^{-1} was observed across the pre-concentration stage (Table 3). As the results were not adjusted for the increase in Brix level during this process, the enhanced patulin level was largely due to the greater concentration of the juice. In addition, raising the temperature during pasteurization significantly reduced mean fungal counts from 4.07 to 0.79 log_{10} cfu ml^{-1}. We postulate that this process may fragment the fungal spores and hyphae present in the juice, which releases patulin and possibly contributes to the observed increase in patulin contamination.

The second part of this study measured the patulin concentrations across the depectinization and ultrafiltration processes, which included the addition of activated charcoal to the depectinization tanks. Patulin concentrations decreased significantly from 110 ± 35 ng ml^{-1} to 75 ± 18 ng ml^{-1} ($P < 0.05$). This was due to the adsorption of patulin by the charcoal as demonstrated in previous studies.[14] Mean *P. expansum* counts did not significantly change during these processes.

Patulin concentrations measured before and after the absorption stage increased from 125 ± 41 ng ml^{-1} to 155 ± 11 ng ml^{-1} while the corresponding *P. expansum* counts remained relatively stable. This apparent increase in patulin levels over the resin-based absorber may be misleading, since the high variability (s.d. ± 41 ng ml^{-1}) among individual samples withdrawn at the pre-treatment stage is indicative of the difficulty of representative sampling. Patulin levels were not significantly altered during the final concentration stage as no real difference was observed between the juice entering and leaving the concentrator. The *P. expansum* counts rose slightly from 0.30 log_{10} cfu ml^{-1} before the concentrator to 1.35 log_{10} cfu ml^{-1}, which may be due to the variation in *P. expansum* counts at the outlet of the concentration unit (0–2.04 log_{10} cfu ml^{-1}).

For legislative, economic and health reasons, the control of patulin in apple juice products is an important consideration in their manufacture. The results of this study re-emphasize the importance of fruit quality. Should the fruit show signs of *P. expansum* contamination, then appropriate initial processing steps must be taken. Both washing and the removal of damaged fruit resulted in a significant decrease in patulin concentration prior to further juice processing. No further significant reduction in patulin was achieved by the routine production of apple juice concentrate, with the exception of the addition of activated carbon and its attendant additional costs.

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