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Stability of patulin in a juice-like aqueous model system in the presence of ascorbic acid

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Abstract

In the present study, the stability of patulin in an aqueous juice-like model system was investigated. At acidic pH, the presence of ascorbic acid reduced the stability of patulin. After 34 days, patulin was reduced to 30% of its initial concentration in the presence of ascorbic acid compared to 68–71% in samples without ascorbic acid. Conditions during storage (presence of light, oxygen and/or metal ions) influenced the stability of patulin. Furthermore, it was possible to induce degradation of patulin by either generating hydroxyl radicals or by adding the rather stable radical diphenyl-1-picrylhydrazyl (DPPH). Data from the present study indicate that patulin is decomposed by free radicals generated by oxidation of ascorbic acid to dehydroascorbic acid. Rapid oxidation of ascorbic acid in the presence of oxygen, catalysed by free metal ions, resulted in a decrease of patulin. After complete oxidation of ascorbic acid, no further patulin degradation was observed. In contrast, slow oxidation of ascorbic acid in the presence of metal-chelators induced a continuous, slow oxidation of patulin. Due to low oxygen content in the headspace of a food package, addition of ascorbic acid to products such as apple juice, prior to filling, cannot be considered as an effective decontamination strategy.

Keywords: Mycotoxins; Patulin; Decontamination; Degradation; Vitamin C; Ascorbic acid

1. Introduction

Patulin is a mycotoxin produced by approximately 60 species belonging to over 30 genera of fungi (Lai, Fuh, & Shih, 2000). It has a broad spectrum of toxicity, including carcinogenicity (Dickens & Jones, 1961) and teratogenicity (Ciegler, Vesonder, & Jackson, 1977) in animals. Symptoms in experimental cases of patulin toxicosis in animals are lung and brain edema, liver, spleen and kidney damage and toxicity to the immune system (Llewelly et al., 1998). According to Lai et al. (2000), for humans, nausea, gastrointestinal disturbances and vomiting have been reported.

As recently reviewed, the most important producer of patulin is the apple-rotting fungus *Penicillium expansum* and apple products may therefore contain patulin when rotten apples have been processed (Drusch & Ragab, 2003). Although exposure assessments have shown that the average exposure of the European population to patulin is well below the maximum tolerable daily intake (0.4 μg/kg body weight) established by the Scientific Committee for Food, specific groups of consumers, especially small children, are more exposed since they tend to consume more apple products.

In order to protect public health, the presence of patulin in food should be reduced to the lowest reasonably achievable level. Therefore, it is necessary to set a maximum level, for those foodstuffs, in which patulin most commonly occurs. The World Health Organisation (WHO) recommends a maximum permitted level of 50 μg/l for apple juice and also the European Commission recently adopted a maximum permitted level of 50 μg/kg for patulin in a range of foodstuffs mainly derived from or containing apples.

In recent decades, research has focussed on the occurrence of patulin in apple juice and in apple juice concentrate (Beretta, Gaiaschi, Galli, & Restani, 2000; Ritieni,

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2003; Yurdun, Omurtag, & Ersoy, 2001), the impact of processing on the reduction of patulin (Kryger, 2001), and technologies for decontamination of apples and apple juice (Gokmen, Artik, Acar, Kahraman, & Poyrazoglu, 2001; Huebner et al., 2000; Leggott, Shephardt, Stockenström, Staal, & van Schalkwyk, 2001). Another important issue, the stability of patulin in fruits and fruit products, as well as factors influencing its stability, remain to be elucidated. The aim of the present study was to investigate the influence of ascorbic acid, as a natural constituent of apples and a commonly used additive in beverages, on the degradation of patulin.

2. Materials and methods

2.1. Materials

Ethyl acetate and acetonitrile, HPLC grade, were obtained from Carl Roth GmbH & Co. KG, Karlsruhe, Germany. Patulin was purchased from Sigma, Deisenhofen, Germany. All other chemicals were purchased from Merck KGaA, Darmstadt, Germany. The water used for all solutions, buffers and HPLC analysis was purified using a Purelab Maxima (USF Elga, Bucks, UK).

2.2. Experimental design

All experiments were performed in a McIlvaine buffer system made from citric acid and disodium hydrogen phosphate. Samples for all experiments were prepared using a standard stock solution of patulin dissolved in ethyl acetate. The organic solvent was removed under nitrogen and redissolved in the different test solutions. Samples for each day of analysis were filled in single screw-cap-sealed test-tubes.

The concentration of patulin was 2 mg/l, the concentration of ascorbic acid was 482 mg/l in the samples. Deactivation of metal ions was achieved by adding ethylenediamintetraacetic acid (EDTA) at a concentration of 0.1 mol/l to the sample. To exclude oxygen in samples the test tubes were purged with nitrogen for 5 min. To prevent exposure to light, samples were stored in the dark.

To induce generation of hydroxyl radicals via the Fenton reaction, ferrous iron and hydrogen peroxide were added to a patulin solution (1200 μg/l). Two different hydrogen peroxide concentrations, 14.7 and 1.47 mmol/l, were chosen. Ferrous iron was added at a level of 10% of the molar concentration of hydrogen peroxide. Diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) was used as a stable radical at a concentration of 0.6 mmol/l.

2.3. Sample clean-up and HPLC analysis

Samples were cleaned up using solid phase extraction according to the method of Trucksess and Tang (2001). Samples were directly passed through a SPE cartridge (Oasis HLB extraction cartridge, 3 cc/60 mg, Waters, Milford, MA) and washed with sodium bicarbonate and acetic acid, both 1%. Patulin was eluted from the cartridge with a mixture of 10% ethyl acetate in ethyl ether solution. The organic solvent was evaporated and immediately redissolved in HPLC grade water (adjusted to pH 4 with acetic acid). All analyses were performed in duplicate. Reverse phase HPLC analysis was performed at room temperature using an Agilent HPLC system including an ALS G1313A autosampler, a G1312A binary pump and a G1315B diode array detector. Elution buffers were: (A) HPLC grade water adjusted to pH 4 with acetic acid and (B) acetonitrile. Separation was achieved under isocratic conditions, using a mixture of 10% B on a Knauer LiChroSpher-60 RP Select B C8 column. Flow rate was 1 ml/min and total run time was 10 min. UV detection was performed at 276 nm.

The method for sample clean-up was compared with the AOAC method 995.10 for liquid chromatographic determination of patulin in apple juice (AOAC 995.10, AOAC Official Methods of Analysis, 1995). In this method, patulin is three times extracted with ethyl acetate, cleaned up by extraction with sodium carbonate and dried with anhydrous sodium sulfate. Ethyl acetate is removed by rotary evaporation and patulin is redissolved in water, adjusted to pH 4 with acetic acid. When analysing a set of patulin-spiked apple juice samples based on normal distribution and *t*-test, no statistical differences between the mean values of the two analytical methods was observed (data not shown). Linearity of patulin determination was confirmed by analysing a diluted patulin standard solution in a concentration range of 24 μg to 20 mg/l sample. The coefficient of determination was $r^2 = 0.9999$. Repeatabilty of the analysis was confirmed by a fivefold clean-up and subsequent analysis of a patulin-spiked buffer solution. At a concentration of 100 μg patulin/l sample, the recovery was 99.7% and the repeatability reflected by the coefficient of variation was 0.71%.

2.4. Determination of ascorbic acid

Ascorbic acid was detected colorimetrically using a commercially available assay of Boehringer Mannheim/r-biopharm (Cat.No. 0409677). The assay is based on the determination of the reduction potential of compounds toward 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) in the presence of an electron carrier at pH 3.5 to a formazan. The MTT-formazan is the measuring parameter and is determined by means of its light absorbance at 578 nm.

3. Results and discussion

3.1. Stability of patulin in aqueous solution at different pH values

To investigate the stability of patulin at different pH values, in a first experiment the concentration was determined

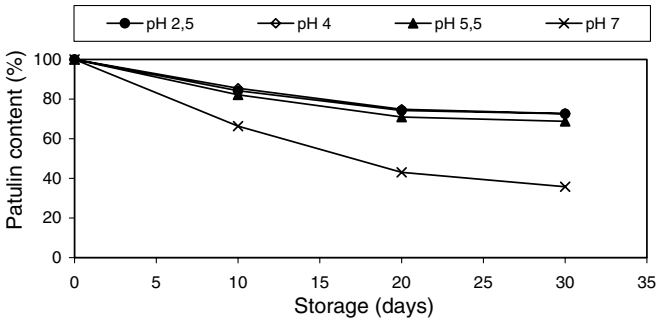

Fig. 1. Influence of pH value on the stability of patulin in aqueous solution.

in McIlvaine buffer, ranging from pH 2.5–7.0. Acidic pH values (2.5, 4 and 5.5) were used as relevant model systems for fruit juices. During a period of 30 days, degradation of patulin in the chosen McIlvaine buffer system was moderate for pH 2.5–5.5. At the 30th day of the experiment, 69–73% of the initial patulin concentration was still present in the samples. At neutral pH, a greater decrease in the patulin concentration to 36% of the initial concentration was observed (Fig. 1). A high stability of patulin, in the range of pH 3.5–5.5, has also been reported by Lovett and Peeler (1973).

3.2. Influence of ascorbic acid and environmental conditions on the degradation of patulin

All following experiments were performed at pH 4.0, which is typical of apple juice. Metal ions and oxygen, as well as exposure to light, were considered to be important factors affecting the stability of patulin. Therefore, the influence of excluding one of these factors, as well as combinations of these factors, was investigated. Fig. 2 compares the degradation of patulin in the presence and absence of ascorbic acid under different storage conditions. In the absence of ascorbic acid, patulin was moderately stable in buffer solution over a period of 34 days. The initial patulin concentration of 2000 μg/l decreased by 32% and 29% under non-protective and protective conditions, respectively. No difference was observed between the samples stored with or without exclusion of light, oxygen and free metal ions. In the presence of ascorbic acid patulin was more rapidly degraded (Fig. 2). After 34 days of storage, patulin was reduced by 66% and 70%. Also, the storage conditions (light, presence of oxygen and no addition of EDTA) caused significant differences in the degradation. After nine days, patulin was reduced by 59% and 37% under non-protective and protective storage conditions, respectively.

Early findings already indicated that different food compounds enhance degradation of patulin. Scott and Somers (1968) reported a rapid degradation of patulin in spiked flour samples and a slow degradation in spiked fruit juices. The authors explained the degradation of patulin via a reaction between patulin and SH-groups. The moderate

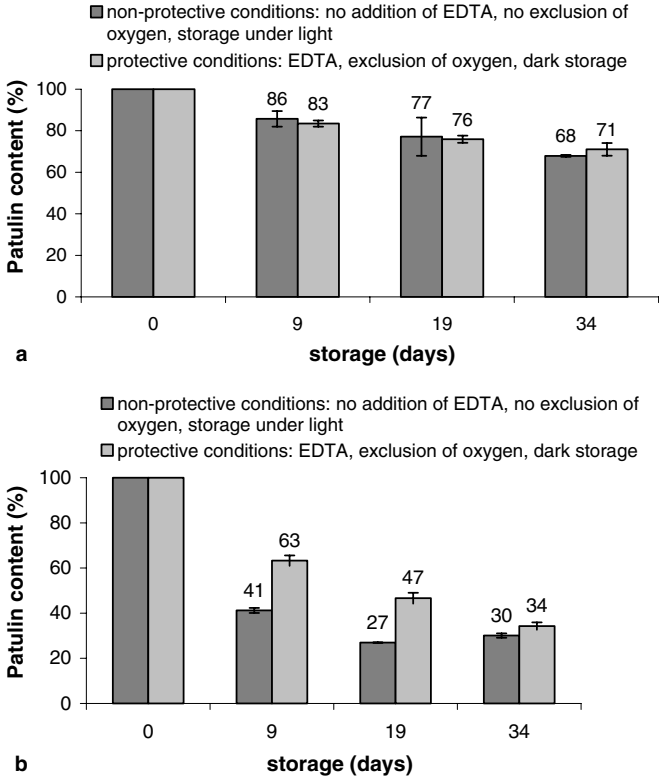

Fig. 2. Degradation of patulin under protective and non-protective conditions (a) without and (b) with addition of ascorbic acid.

stability of patulin in apple and grape juice was attributed to very low levels of sulfhydryl groups. Brackett and Marth (1979) first observed a reduction of patulin by ascorbic acid and postulated a possible mechanism of patulin degradation. The authors implied a metal-catalysed oxidation of ascorbic acid or ascorbate resulting in the generation of singlet oxygen or free radical forms of a metal ascorbate complex as described by Martell and Kahn (1973).

Data from the present study (Fig. 2) indicate that both, free radicals, such as hydroxyl radicals or singlet oxygen, as well as a free radical metal ascorbate complex, are involved in the degradation of patulin.

Free radicals result from metal ion catalysed oxidation of ascorbic acid in the presence of oxygen, whereas a free radical metal ascorbate complex results from metal chelate-catalysed oxidation of ascorbic acid. The latter reaction is not dependent on the presence of oxygen. Martell and Kahn (1973) indicated that metal ion catalysis of the oxidation of ascorbic acid is a reaction in which oxygen is reduced to peroxide or water and the metal ion serves as an electron transfer agent between the substrate and the oxidant (Fig. 3). Hydroxyl radicals may be generated from hydrogen peroxide in the presence of metal ions according to the Fenton reaction. Singlet oxygen may result from a spontaneous dismutation of superoxide (Fridovich, 1974).

In the present study, ascorbic acid was completely oxidised after nine days of storage under non-protective conditions (data not shown). As a consequence, free radicals

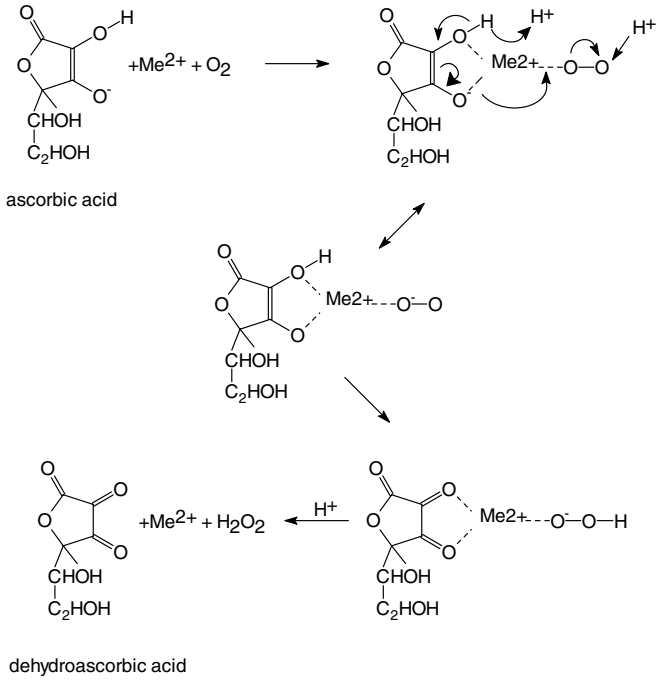

Fig. 3. Mechanism of metal-catalysed oxidation of ascorbic acid (adopted from Martell and Kahn (1973)).

were present and a fast reduction of patulin occurred, which levelled off after nine days.

In contrast, degradation of ascorbic acid was slow in the sample stored under protective conditions (dark storage, nitrogen, chelation of metal ions). After 34 days of storage, 65% of ascorbic acid was still present in the sample. It seems likely that in this case, ascorbic acid underwent a metal chelate-catalysed oxidation, as shown in Fig. 4. The rate-limiting step is the reduction of metal ions and the formation of semiquinone-like radicals. In the present study, these semiquinone-like radicals were responsible for patulin degradation. Since ascorbic acid was still present in sufficient amount, it seems likely, that patulin degradation in the sample would proceed upon further storage.

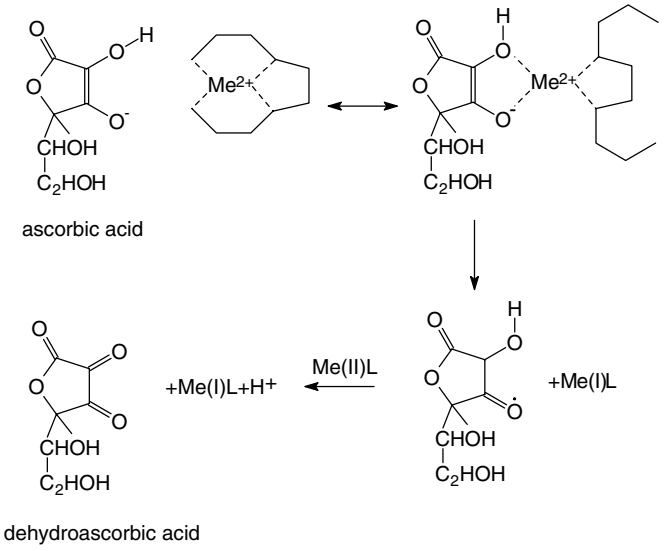

Fig. 4. Mechanism of metal chelate-catalysed oxidation of ascorbic acid (adopted from Martell and Kahn (1973)).

To flesh out the two step mechanism of oxidation of ascorbic acid with subsequent degradation of patulin by free radicals, in the present study patulin degradation was induced by generating free radicals through addition of hydrogen peroxide and ferrous iron or by direct addition of the stable radical, DPPH·. A concentration of 0.05% hydrogen peroxide was chosen, since it was reported to be efficient at that concentration in removing citrinin from feed (Bischof, 1999). As it is to be seen in Fig. 5, it was possible to induce a rapid degradation of patulin through the addition of a combination of hydrogen peroxide and ferrous iron. In samples, which were analysed immediately after adding hydrogen peroxide and ferrous iron, patulin was reduced to 26% of its initial concentration. Similar results were obtained when using DPPH· for the induction of patulin degradation. Immediately after sample preparation only 57% of patulin was detected. Compared to radicals generated via the Fenton reaction, using hydrogen peroxide, the reactivity of DPPH· is low and may explain the difference between the samples. When using 0.005% hydrogen peroxide, no differences in the patulin concentration over time were observed between samples with added hydrogen peroxide, with added hydrogen peroxide plus ferrous iron or the control group without any additives.

3.3. Influence of environmental conditions on the degradation of patulin

The results of patulin degradation in the presence of ascorbic acid in the present study (Fig. 2) are in agreement with other data reported in the literature (Table 1). However, rates of reduction of patulin as a function of time, initial concentration of patulin and ascorbic acid concentration and environmental conditions vary between all studies.

Steiner, Werner, and Washüttl (1999) concluded from their results that, by increasing the concentration of ascorbic acid, a higher rate of patulin degradation can be achieved. Taking into account other data, more influencing factors become obvious. Steiner et al. (1999) reported a

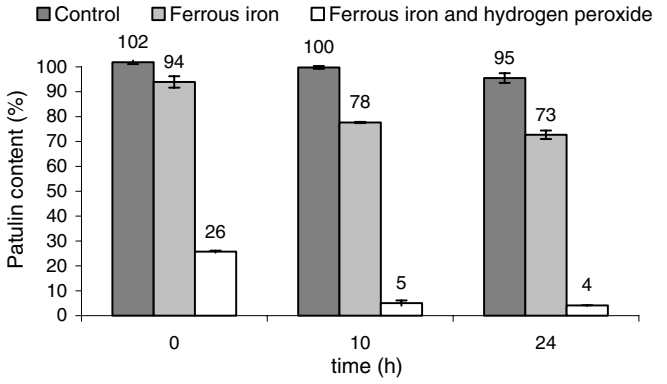

Fig. 5. Degradation of patulin by addition of hydrogen peroxide and ferrous iron over a period of 24 h.

Table 1
Available data on the degradation of patulin in the presence of ascorbic acid

Type of sample	Patulin concentration (μg/l)	Ascorbic acid addition (mg/l)	Storage (days)	Storage conditions	Degradation (%)	Reference
Canned apple juice	4000	0	21	Dark storage	20–25	Scott and Somers (1968)
Aqueous buffer solution, pH 3.5	5000	0	8	Unknown	20	Brackett and Marth (1979)
	5000	30,000	8		80	
Aqueous buffer solution, pH 4.0	5,000,000	0	15	Dark storage	20	Aytac and Acar (1994)
	5,000,000	500	15		100	
Apple juice	3,000,000	35	60		83	
	3,000,000	35	120		98	
Apple juice	500	150	8/20	Unknown	65/100	Valletrisco et al. (1991)
	500	300	8/16		50/90	
	800	150	8/20		78/100	
	800	300	8/16		70/94	
Apple juice, clear	57	103	14	Dark storage under N_2	61	Steiner et al. (1999)
	57	499	14		64	
	57	999	14		97	
Apple juice, cloudy	82	103	14	Dark storage under N_2	45	Steiner et al. (1999)
	81	501	14		35	
	81	999	14		64	

reduction of 50 μg patulin/l in apple juice of 64% through the addition of 500 mg ascorbic acid/l within 14 days. In contrast, Aytac and Acar (1994) achieved a complete degradation of 5000 mg patulin/l with 500 mg ascorbic acid/l during 15 days. The reduction in patulin content reported by Steiner et al. (1999) is also considerably lower than the reduction reported by Valletrisco, De Clemente, Niola, and Casadio (1991) or Brackett and Marth (1979). The difference is probably due to the availability of oxygen in the samples, which has been excluded in the study of Steiner et al. (1999).

Fig. 6 demonstrates the single effects of deactivation of metal ions, exclusion of oxygen or prevention of exposure to light. The influence of metal ions was tested in the presence of light and oxygen through deactivation of metal ions. Through storage in the dark, the influence of exclusion of light in the presence of metal ions and oxygen was investigated and through purging with nitrogen the influence of oxygen in the presence of light and metal ions was tested.

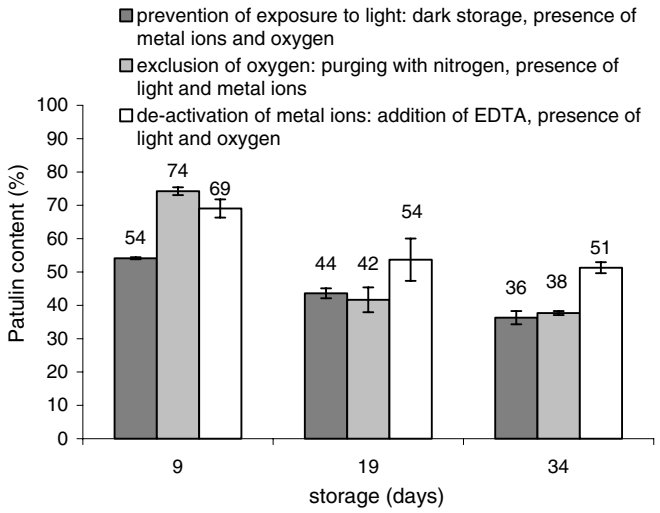

Fig. 6. Degradation of patulin in the presence of ascorbic acid and deactivation of metal ions, exclusion of light or prevention of exposure to light.

After nine days of storage with exclusion of oxygen, i.e., in the presence of metal ions and light, 74% of patulin was still detectable in the sample compared to 41% in the sample stored under the influence of oxygen, light and metal ions, as shown in Fig. 2. By purging the samples with nitrogen, oxygen was probably not completely removed from the sample. At ambient temperature, maximum level of dissolved oxygen in water is approximately 9 mg/l. Remaining oxygen was obviously sufficient to induce oxidation of ascorbic acid and subsequent generation of free radicals.

Exclusion of metal ions by chelation with EDTA also retarded degradation of patulin and therefore supports the metal-chelate-induced oxidation of ascorbic acid described above. The presence of light showed the lowest impact on patulin degradation. After nine days of storage in the dark, only 54% of the initial patulin concentration was detected, while the concentration of ascorbic acid was even higher than in the other samples. It therefore seems likely that, in the presence of oxygen, the oxidation of ascorbic acid is only necessary for initiating a free radical generation via the Fenton reaction.

Efficient decontamination strategies for patulin-contaminated foods such as apple juice and apple juice concentrate do not exist. Lovett and Peeler (1973) showed that thermal processing of low-acid foods may not destroy patulin. In the presence of sulfhydryl groups or sulfite, patulin is rapidly degraded (Aytac & Acar, 1994; Fliege & Metzler, 2000). As reviewed by Steiner et al. (1999), at acidic pH a reversible binding of sulfite to patulin occurs. The resulting hydroxysulfonate still includes the conjugated lactone ring, which is the toxicologically relevant structure. Furthermore, due to its allergenic potential, the use of sulfite in apple juice, which is frequently consumed by infants and young children, is not recommended.

Brackett and Marth (1979) suggested that ascorbic acid may be an efficient additive for decontamination of apple juice. Data from the present study indicate that, for a rapid degradation of patulin by ascorbic acid, the presence of oxygen and free radicals is necessary. Oxidation of ascorbic acid in the presence of oxygen and metal ions is a possible source of these radicals. Since patulin degradation levelled off after complete oxidation of ascorbic acid, the initial concentration of ascorbic acid and its rate of degradation are important factors. Due to low oxygen content in the headspace of a food package, addition of ascorbic acid to products such as apple juice, prior to filling, is not an effective decontamination strategy. Furthermore, the toxicological potential of the resulting patulin degradation products remains to be clarified.

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