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D. Spadaro*, A. Ciavorella, S. Frati, A. Garibaldi, M.L. Gullino

AGROINNOVA, Centre of Competence for the Innovation in the Agro-environmental Sector, University of Turin, Via L. da Vinci 44, 10095 Grugliasco (TO), Italy

*Corresponding author. Tel.: +39-011-6708942; fax: +39-011-6709307.

E-mail address: davide.spadaro@unito.it (D. Spadaro)

Abstract

A survey on the occurrence of patulin was conducted during 2005 on commercial pure apple juices (53 samples) and mixed apple juices (82 samples) marketed in Italy. The current study was undertaken to investigate the possible influence of the agro-food production process employed (conventional or organic), of the fruit percentage in the commercial product (higher or lower than 50%) and of the type of apple juice (clear or cloudy) on the occurrence and level of patulin contamination. Patulin could be quantified in 34.8% of the samples ranging from 1.58 to 55.41 μg kg^{-1}. With the exception of one sample, the level of patulin was lower than 50 μg kg^{-1}, the maximum permitted threshold in fruit juices according to the European legislation. Mean levels of patulin were significantly lower in mixed apple juices (4.54 μg kg^{-1}) than in pure apple juices (9.32 μg kg^{-1}). Levels of patulin contamination were comparable in clear and cloudy juices. A similar incidence of positive samples was found in conventional and organic apple based juices, and the magnitude between the mean contamination levels, although higher in organic (10.92 μg kg^{-1}) than in conventional juices (4.77μg kg^{-1}), was not statistically significant (P=0.771; Mann-Whitney test). The magnitude between the means of patulin contamination in juices containing more than 50% fruit (11.26 μg kg^{-1}) and in juices with 50% or less fruit (3.35 μg kg^{-1}) was statistically significant

(P=0.016; Mann-Whitney test).

Key words: Apple juice, HPLC, Mycotoxin, Patulin, *Penicillium expansum*.

1. Introduction

Patulin (4-hydroxy-4H-furo-[3,2-c]pyran-2(6H)-one) is a secondary metabolite produced by some species of *Aspergillus*, *Byssochlamys* and *Penicillium* (Weidenbörner, 2001). Apples and apple products are excellent substrates for *Penicillium expansum*, the causal agent of blue mould, to produce the mycotoxin. The fruit pathogen is generally associated with damaged fruit or fruit already infected by other microorganisms in orchard as well as in postharvest conditions (Snowdon, 2001).

Patulin contamination of apple juice is an effective indicator of unsound rotted apples in juice manufacture. Removal of decayed and damaged fruit or trimming of mouldy portions can significantly reduce patulin levels in apple products (Lovett, Thompson & Boutin, 1975).

Acute symptoms of patulin consumption can include agitation, convulsions, edema, ulceration, intestinal inflammation and vomiting (Speijers, 2004). Chronic health effects of patulin include genotoxicity, immunotoxicity, and neurotoxicity in rodents, while its effects on humans are not clear yet (Wouters and Speijers, 1996).

The Joint FAO/WHO Expert Committee on Food Additives (JEFCA) established a provisional maximum tolerable daily intake (PMTDI) of 0.4 μg kg^{-1} body weight (bw) day^{-1}, based on a no observable effect level (NOEL) of 43 μg kg^{-1} bw day^{-1} and a safety factor of 100 (World Health Organisation, 1995).

The maximum permitted level of patulin in fruit juices and nectars, in particular apple juices and apple juice ingredients in other beverages marketed in Europe is 50 μg kg^{-1} (European Commission, 2003). The permitted threshold is lower for apples juices labelled and sold as intended for infants and young children (10 μg kg^{-1}). Several studies have demonstrated the contamination of patulin in apple juices of different countries, including Australia, Austria, Belgium, Brazil, Canada, France, Iran, Italy, Japan, South Africa, Spain, Sweden, United Kingdon, United States, Turkey (Moake, Padilla-Zakour & Worobo, 2005).

Previous studies have evaluated the patulin content in apple derivatives commercialized in Italy. Beretta, Gaiaschi, Galli & Restani (2000) analysed 26 apples, 23 homogenized baby-foods, 21 clear apple juices and 12 cloudy apple juices, finding that, in apple juices and homogenised baby-foods, the mycotoxin concentration was always below the established limits of 50 and 10 μg kg^{-1} respectively. In a study published in 2003, 40 apple based products were analysed, including 15 apple juices and 6 apple and other fruit juices (Ritieni, 2003). The mixed juices analysed were patulin-free while 6 of the apple juices were contaminated at levels ranging from 1.4 to 56.4 μg kg^{-1} of the mycotoxin. During November 2003 – February 2004, 169 samples purchased in Italian markets, supermarkets and organic food shops, including 57 apple juices, 15 pear juices and 57 other juices, were analysed (Piemontese, Solfrizzo & Visconti, 2005). Sixteen of the 33 conventional apple juices were contaminated, as well as 12 of the 24 organic apple juices.

In this study we concentrated on the occurrence and level of patulin in apple based juices, containing 100% apple juice or a certain percentage of apple juice together with other fruit juices. The last category represents most of the apple based juices marketed in Italy. A second aim of the work, was the investigation of the possible influence of the agricultural production process employed (conventional or organic), of the fruit percentage in the commercial product (higher or lower than 50%) and of the type of apple juice (clear or cloudy) on the occurrence and level of patulin contamination.

2. Materials and Methods

2.1. Sample preparation

Commercial fruit juices (135 samples) were purchased at random from Italian supermarkets or organic food shops during the period April – November 2005. They represent all types and brands of Italian and imported products sold in Italy. They were classified based on composition (pure apple juices / mixed juices with a percentage of apple juice), agricultural production methods (conventional / organic), and fruit percentage (50% or less / more than 50%).

The extraction procedure used, modified by Arranz, Stroka, Kroeger, Mischke, & Anklam (2004), permitted to quantify 10 μg kg^{-1} or lower levels of patulin. Cloudy juices were left overnight at room temperature or 2h at 40 °C with pectinase enzyme solution (Sigma Chemical Co., St Louis, MO, USA; 5 U / g of juice) and then centrifuged at 4500 rpm for 5 min. Thirty g of clarified juice were extracted with ethyl acetate (Sigma Chemical Co.). The organic phase was dehydrated with 15 g of sodium sulphate anhydrous (Merck, Darmstadt, Germany) and then evaporated to dryness (Rotavapor Laborota 4000, Heidolph®, Schwaback, Germany).

The clean-up was performed modifying the procedure of Stray (1978). The sample was dissolved in 10 ml of toluene and 5 ml of sample were cleaned-up with C_{18} SPE column (100mg, 6ml, J.T. Baker®, Phillipsburg, NJ, USA) previously trigged with 5 ml of toluene (Sigma Chemical Co.). The column was washed with toluene (2 ml) and the sample was eluted with 4 ml of toluene: ethyl acetate (1:1). The final eluate was evaporated to dryness (Reack-Therm III, Pierce®, Rockford, IL, USA), dissolved with 1.5 ml of acetic acid solution (4.35 mM, pH 4.0), filtered through a 0.22 μm syringe filter (Millipore®, Bedford, MA, USA) and transferred into a HPLC vial.

2.2. HPLC-DAD analysis

The HPLC apparatus was an Agilent 1100 series equipped with G 1379 degasser, G 1313A autosampler, G1316A column thermostat set at 30°C, G 1315B UV diode array detector set at 276 nm, G1311 quaternary pump and Agilent Chemstation G2170AA Windows XP operating system (Agilent®, Waldbronn, Germany). A stainless steel analytical column (250 x 4.6 mm i.d., 4 μm, Synergy Hydro-RP C18; Phenomenex®, Torrance, CA, USA) preceded by a guard column (4 x 3 mm i.d.) with the same stationary phase was used. The mobile phase, eluting at a flow rate of 1 ml/min, consisted of an isocratic mixture of water-acetonitrile-perchloric acid (96:4:0.1) for 16 min, followed by a washing step with an isocratic mixture of water-acetonitrile (35:65). 100 μl of sample were injected onto the HPLC column and the retention time of patulin was 11.82 min.

The amount of patulin in the final solution was determined by using a calibration graph of concentration versus peak area and expressed as ng/ml, achieved by injection onto the HPLC column of 100 μl of standard solutions of patulin (Sigma Chemical Co.) prepared according to the method described by Arranz, Stroka, Kroeger, Mischke, & Anklam (2004). The standard solutions had concentrations of 500 ng ml^{-1}, 400 ng ml^{-1}, 250 ng ml^{-1}, 100 ng ml^{-1} and 50 ng ml^{-1} of patulin.

2.3. In-house validation

The recovery was determined on a blank fruit juice spiked at three concentrations of patulin (8, 30 and 50 μg kg^{-1}). Each test was performed three times and the mean recovery values were respectively 94.0, 91.9 and 93.2%. The repeatability ranged from 4.7 to 7.7% for triplicate analyses.

The chromatogram (figure 1) clearly shows the absence of interfering signals at the patulin retention time, as well as an efficient separation from 5-hydroxymethylfurfural (5-HMF), a common compound of apple juice resulting from the breakdown of simple sugars (such as glucose or fructose) at pH 5 or lower, eluting just prior to patulin.

The limit of detection (LOD) and the limit of quantification (LOQ), based on the IUPAC definition (Thompson, Ellison, & Wood, 2002), were respectively 1.04 and 1.57 μg kg^{-1}. The high value of the regression coefficient ($R^2 \geq 0.99$) obtained indicated a good linearity of the analytical response.

2.4. Statistical analysis

Samples with a concentration of patulin higher than the LOQ were considered positive, whereas samples with concentrations between the LOD and the LOQ were considered negative. Mean patulin concentrations were calculated by using LOQ/6 for negative samples according to Majerus & Kapp (2002). Experimental results are reported as mean ± standard deviation, median and maximum. The Mann-Whitney test was used to compare the mean patulin levels in apple and mixed juices, in traditional and organic juices, in juices containing more or less that 50% fruit concentrate and in clear and cloudy apple juices, using the null hypothesis that the two levels were not different. The χ^2-test was used to compare the patulin contamination frequencies of apple and mixed juices, of clear and cloudy apple juices, and of juices containing more or less that 50% fruit. Statistical analyses were performed by using the programme SPSS Release 12.01 (2003).

3. Results and discussion

Recent surveys on patulin occurrence concerned apples, pure apple juices, pure apple purees, or apple ciders (Beretta et al., 2000; Tangni, Theys, Mignolet, Maudoux, Michelet & Larondelle, 2003; Boonzaaijer, Bobeldijk, & van Osenbruggen, 2005), whereas few reports are available on the occurrence of patulin in mixed juices containing apple and other fruit juices (Leggott & Shephard, 2001; Ritieni, 2003; Piemontese et al., 2005). Leggott & Shephard (2001) analysed 25 mixed fruit juices and purees, finding 6 positive samples. Ritieni (2003) analysed 6 mixed apple juices and all of them resulted negative. Piemontese et al. (2005) analysed 57 samples of "other" juices, including fruit juices other than apple and pear or juices containing apple together with other fruit.

This research focused on apple-based juices, containing only apple juice (53 samples) or a certain percentage of apple juice mixed with other fruit juices (82 samples). The last category includes most of the apple based juices marketed in Italy. To our knowledge, this is the first investigation performed on a significant number of mixed apple juices.

Patulin could be quantified in 47 out of 135 pure apple or mixed apple juices (ranging from 1.58 to 55.41 $\mu g\ kg^{-1}$). An overall incidence of 34.8% was observed in the apple based juices, with 24 samples having between 1.57 $\mu g\ kg^{-1}$ (LOQ) and 10 $\mu g\ kg^{-1}$ patulin, 22 samples having between 10 $\mu g\ kg^{-1}$ and 50 $\mu g\ kg^{-1}$ patulin, and one sample exceeding the 50 $\mu g\ kg^{-1}$ patulin threshold (Table 1). A mean contamination level of 6.42 $\mu g\ kg^{-1}$ was calculated for all contaminated samples.

According to the typology of juices, the magnitude between the means of patulin level in pure apple juices (9.32 μg kg^{-1}) and mixed apple ones (4.54 μg kg^{-1}) was statistically significant ($p = 0.012$, Mann-Whitney test). Also the medians of the two juice typologies were significantly different, respectively 1.39 μg kg^{-1} and 0.27 μg kg^{-1}. A patulin incidence of 47.2% was registered in pure apple juices, while a lower occurrence (26.8%) resulted in mixed apple juices. The χ^2-test showed that the frequencies of patulin occurrence in pure apple and mixed apple juices were not comparable ($p = 0.0003$). Although higher incidence and level of contamination were found in pure apple juices, also mixed apple juices have a significant mean patulin contamination. The sample with the highest patulin contamination, exceeding the limit of 50 μg kg^{-1} was an organic mixed apple one (55.41 μg kg^{-1}). Probably, the relative high contamination found in mixed juices could be explained with a lower attention to the quality of the single juice added to the mixture: mixed juices generally contain higher quantities of sugars and other additives.

The results (Table 1) also show a comparison of the mean patulin contamination level in clear (10.81 μg kg^{-1}) and cloudy (7.59 μg kg^{-1}) apple juices. Such division was possible only for pure apple juices, because all mixed apple juices purchased and analysed in this study were cloudy. The hypothesis that the mean patulin contamination levels in clear and cloudy apple juices were not different was accepted ($p = 0.940$; Mann-Whitney test), as already shown in previous studies (Tangni et al., 2003). A similar incidence of patulin contamination was registered in clear (50.0%) and cloudy juices (44.0%). Moreover, the χ^2-test showed that the frequencies of patulin occurrence in clear and cloudy apple juices were comparable ($p = 0.356$). This finding suggests that the clarification of apple juice probably did not significantly change the level of patulin contamination in clear juices compared to cloudy ones, though previous studies (Stray, 1978) highlight the possible reduction of about 20% of patulin with standard juice clarification processes.

According to a study carried out by Beretta et al. (2000), organically produced apple juices are more contaminated by the mycotoxin than conventionally produced ones. Ritieni (2003) and Tangni et al. (2003) compared organic and conventional produced apple juices without finding any statistically significant difference. Piemontese et al. (2005) showed a statistically higher incidence of positive samples and mean patulin concentration in organic products as compared to conventional ones. On the other hand, a similar incidence of positive samples was found in conventional and organic apple juices, with mean patulin concentrations statistically not different.

In this study (Table 1), a similar incidence of positive samples was found in conventional (35.7%) and organic (32.4%) apple based juices, although the mean contamination level in organic juices (10.92 μg kg^{-1}) was double the value found in conventional juices (4.77 μg kg^{-1}). The two medians can add some information because they are quite similar. The hypothesis that the mean patulin contamination levels in conventional and organic apple juices were not different was accepted (p = 0.771; Mann-Whitney test). Even narrowing the statistical analysis to the pure apple juices, no significant difference can be registered between the mean patulin contaminations in conventional (8.96 μg kg^{-1}) and organic (9.91 μg kg^{-1}) pure apple juices (p = 0.336). The fact that no significant differences were registered between organic and conventional fruit juices could be explained with the same care used in both production chains in removing decayed and damaged fruit during juice processing.

Regarding the fruit content, the juices analysed were divided in two classes (Table 1): juices with fruit content declared on the label of 50% or lower (ranging from 25 to 50%) and juices with more than 50% fruit content (ranging from 55 to 100%). The first category included most of the samples analysed in this study. The magnitude between the means of patulin contamination in juices containing more than 50% fruit (11.26 μg kg^{-1}) and in juices with 50% or less fruit (3.35 μg kg^{-1}) was statistically significant (p = 0.016; Mann-Whitney test). An overall incidence of 48.1% was observed in juices with more than 50% fruit against 26.5% for the juices with 50% or less fruit. The χ^2-test showed that the frequencies of patulin occurrence in juices with higher and lower fruit content were not comparable ($p = 1.4 \times 10^{-9}$). Low fruit content juices have significantly lower patulin contamination mean and incidence. Although these data are quite predictable, the study constitutes one of the first evidences about the different level of patulin contamination in juices with different fruit content.

In conclusion, most of the data shown in the present study indicate an acceptable situation, with a low level of contamination in the pure or mixed apple juices marketed in Italy. With the exception of one sample, the level of patulin was lower than 50 μg kg^{-1}, the maximum permitted threshold in fruit juices according to the European legislation.

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Figure 1

Representative HPLC separation of patulin and 5-HMF in a clear apple juice spiked with 50 µg patulin kg^{-1}

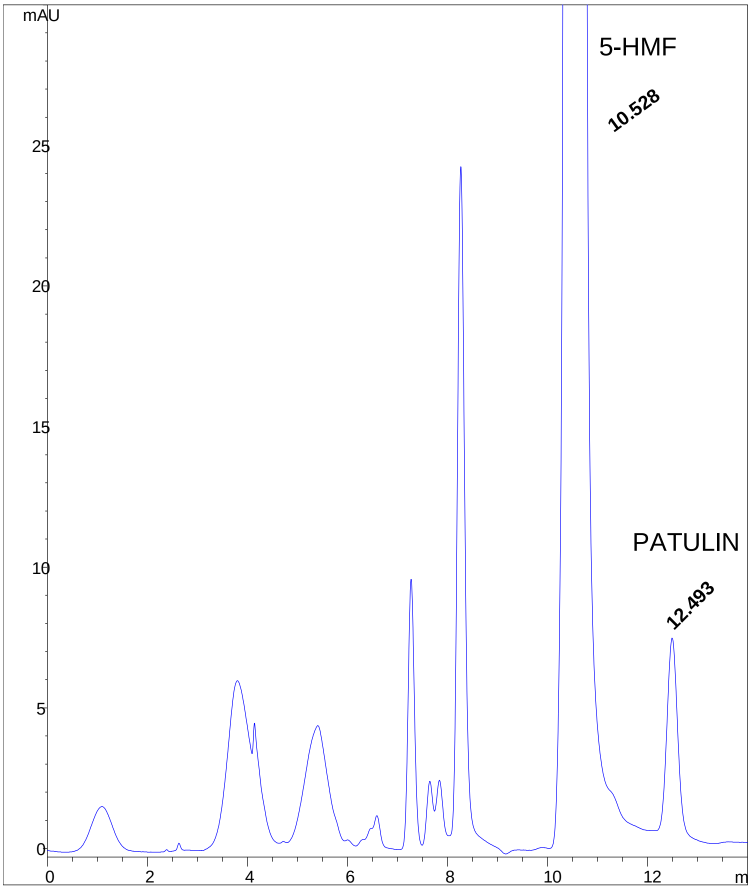

Table 1

Patulin contamination in juices containing 100% apple juice or a certain percentage of apple juice together with other fruit juices, marketed in Italy

Commodity	**Positive / total**	**Positive (%)**	**Number of samples**			**Mean* ± SD (μg kg^{-1})**	**Median (μg kg^{-1})**	**Max (μg kg^{-1})**
			<10 μg kg^{-1}	**10-50 μg kg^{-1}**	**>50 μg kg^{-1}**			
Apple juices	25/53	47.2	13	12	-	9.32±5.07	1.39	47.91
Clear apple juices	*14/28*	*50.0*	*8*	*6*	-	*10.81±4.27*	*2.17*	*47.91*
Cloudy apple juices	*11/25*	*44.0*	*5*	*6*	-	*7.59±5.62*	*1.06*	*44.89*
Mixed juices	22/82	26.8	11	10	1	4.54±3.88	0.27	55.41
Conventional juices	35/98	35.7	20	15	-	4.77±3.32	0.77	44.89
Organic juices	12/37	32.4	4	7	1	10.92±6.37	0.80	55.41
Conventional apple juices	*19/32*	*59.4*	*11*	*8*	-	*8.96±4.46*	*2.73*	*44.89*
Organic apple juices	*6/21*	*28.6*	*2*	*4*	-	*9.91±5.91*	*0.80*	*47.91*
Juices with more than 50% fruit content [1]	25/52	48.1	13	11	1	11.26±5.13	1.31	55.41
Juices with 50% or less fruit content [2]	22/83	26.5	11	11	-	3.35±1.71	0.69	44.89
Total juices	**47/135**	**34.8**	**24**	**22**	**1**	**6.42±4.48**	**0.77**	**55.41**

*Mean level was calculated using LOQ/6 for negative samples. [1] Fruit content: mean 93.5%, range 55-100%. [2] Fruit content: mean 41.8%, range 25-50%.