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Patulin in Italian Commercial Apple Products

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Patulin is a mycotoxin produced by microscopic fungi belonging to the *Penicillium* and *Aspergillus* genera. The natural occurrence of patulin in four apple products marketed in Italy and purchased from the supermarket, herbalist, and retail shops was studied. Thirty-three samples of the four products had no detectable patulin contamination. The 11 positive samples had a concentration ranging between 1.4 and 74.2 μg/L with a mean of 26.7 μg/L. All vinegar samples were negative for patulin; of 10 apple-based baby foods, two samples were contaminated with 17.7 and 13.1 μg/L and both were labeled as "organic food". Comparing organic and conventional agricultural practices, no significant differences were found. Finally, optimization of extraction protocol more general and useful for juices, clarified juices, baby foods, vinegars, and purees was performed. The low incidence of the patulin level in Italian apple products is a clear parameter to judge the quality of the fruit, and the process is of a high standard.

KEYWORDS: Patulin; apple juice; mycotoxins

INTRODUCTION

Mycotoxins are secondary bioactive metabolites produced mainly by the mycelial structure of filamentous fungi, or more specifically, the molds. The biochemical significance of mycotoxins in fungal growth and development is not clear yet (*1*). Patulin (4-hydroxy-4H-furo[3,2-c]pyran-2[6H]one) (**Figure 1**) is considered one of the most important mycotoxins, and it is biosynthesized by several species of filamentous fungi belonging to the genera *Penicillium*, *Aspergillus*, and *Byssochlamys* (*2*, *3*). Patulin, considered an unwanted natural contaminant of fruits, can be used as an indicator of the quality of fruits and related transformation processes (*4*, *5*). Patulin has been evaluated in apples and their products and sometimes in cereals, bread, pears, apricots, peaches, grapes and products derived from these fruits (*6*).

Penicillium expansum is considered the most important producer of patulin (*7*), and it is commonly identified as the "blue mold rot" found in storage-rotted apples (*8*, *9*). *P. expansum* develops often on the surface of healthy fruit (*10*) but is normally associated with damaged fruits or fruits already infected by other microrganisms in orchards and postharvest conditions (*11*, *12*).

Thermal processing appears to cause only a moderate reduction in patulin levels; thus, the patulin present in apple juice will survive the usual pasteurization processes, addition of ascorbic acid and ascorbate, irradiation, and addition of activated charcoal and sulfur dioxide (*13*).

Patulin is chemically a lactone, relatively stable under acidic pH (*14*), and related to 6-methylsalicilic acid (*15*) with antibiotic properties and an LD_{50} (ip) in mice of 5 μg/kg (*16*). Patulin has been reported to be mutagenic, genotoxic, immunotoxic, and neurotoxic in rodents, but its effects on the human are not clear yet (*17*). Several studies from different countries revealed moderate patulin incidence but at low levels (*18*−*20*). An exception has been the high level of patulin observed in some studies (*4*, *21*). In 1993, a series of reports in the U.K. from the Ministry of Agriculture, Fisheries, and Food (*22*) were kicked off to survey patulin occurrence in apples and their derivates. Other studies of the MAFF in 1980 and 1984 showed low patulin occurrences at 38 and 56 μg/kg, respectively (*13*). In 1993, this level was found to be 118 μg/kg and four samples were contaminated at 434 μg/kg (*22*). In 2003, the Food Standards Agency survey has found three samples of pressed apple juice above the proposed limit of 50 μg/kg for patulin (*23*). After these reports, several countries, including the U.K., South Africa, etc., have recommended a residual patulin concentration of less than 50 μg/kg for apple for human consumption. This level has been reduced to 20 and 30 μg/kg for infant and children foods (*24*, *25*). Finally, the Joint Food Organization Expert Committee on Food Additives suggests a provisional tolerable weekly intake of 7 μg/kg body weight/week (*26*). Patulin surveys in fruit juice and their products have been performed in several countries such as Australia, U.K., South Africa, Turkey, and Brazil (*18*−*20*).

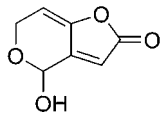

Figure 1. Chemical sketch of patulin.

In this paper, a comparison of three procedures to extract patulin from apple juice, cleared apple juice, infant food, and

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Table 1. Brief Resume of Sample Features

sample	quantity	agronomic practice claimed	comments
apple juice 100%	7	conventional	one of them was claimed GMO
apple juice with other fruits	4	conventional	one of them was claimed GMO
apple juice 100%	7	organic	
apple juice 100%	1	integrated	
apple juice with other fruits	2	organic	
baby food with apples	6	conventional	
baby food with apples	4	organic	
apple vinegar	2	organic	
apple vinegar	1	conventional	
apple puree	1	conventional	with antifermentative
apple puree	1	conventional	without antifermentative
apple puree	1	organic	with antifermentative
apple puree	1	organic	without antifermentative
apple puree	1	integrated	with antifermentative
apple puree	1	integrated	without antifermentative

apple vinegar and a survey conducted on the potential patulin contamination products marketed in Italy are described. In addition, a comparison between organic and conventional apple products and industrial purees obtained from organic, integrated, and conventional apples with and without antifermentative additives were performed.

MATERIAL AND METHODS

Patulin standard was purchased from Sigma Chemical Company (St. Louis, MO). Water for the high-performance liquid chromatography (HPLC) mobile phase was purified in a Milli-Q system (Millipore, Bedford, MA). All other chemicals and solvents were HPLC grade from Merck (Darmstadt, Germany).

Sampling. From a supermarket, local stores, an herbelist, and salesrooms in the Naples area, 40 samples (two subsamples for each lot) of retail pure apple juice, apple juice mixed with other fruits, apple purees, apple vinegar, apple-based baby foods, and apple mousse were purchased. Industrial purees were kindly supplied by the Italian juice industry, and it supplied the partial process layout too.

The fruit juices were in glass or plastic bottles, tetra brik, or drink easy open cans. Concentrated purees were diluted (1:1 v:v) with distilled water prior to analysis. All of the samples were stored at 4 °C in the dark prior to analysis by HPLC. In **Table 1** is a brief description of the collected samples.

Preparation of Patulin Working Solution. Patulin standard stock solution was prepared by dissolving 1 mg of pure standard into 1 mL of methanol HPLC grade. One hundred microliters of patulin standard stock solution (1 mg/mL) was transferred into a 10 mL volumetric flask and completely dried under nitrogen stream at room temperature. It was quickly diluted with acetic acid solution (acetic acid solution, 30% w/w density, 1.04 g/mL, 5 M, pH 4, from Merck) to obtain a working solution at 10 000 μg/L standard working solution and then diluted with acetic acid solution at pH 4. The vials were closed and stored in the dark at 4 °C; the working solution was freshly prepared every week.

Extraction Procedure. *Method A.* According to the procedure described by Malone et al. (*27*), 10 g of apple products was homogenized with 50 mL of ethyl acetate for 3 min at 13 500 in an Ultraturrax. The corresponding homogenate was centrifuged at 2000*g* for 3 min at 4 °C (RC 10.10, Jouan S. A., St. Herblain, France). The upper part of the centrifuge (40 mL), carefully collected by funnel, was evaporated under reduced pressure at 40 °C. The organic residue was suspended in 1 mL of acetic acid solution at pH 4, and then, 20 μL was injected onto an HPLC column for analysis.

Method B. According to the procedure described by Sewram et al. (*28*), 10 g of apple products was diluted with 10 mL of pure water and mixed. This solution was extracted three times with 20 mL of ethyl acetate, and at the organic phase, sodium sulfate was added dry, filtered, and completely dried under reduced pressure at 40 °C. The organic residue was suspended in 1 mL of acetic acid solution at pH 4, and then, 20 μL was injected onto an HPLC column for analysis.

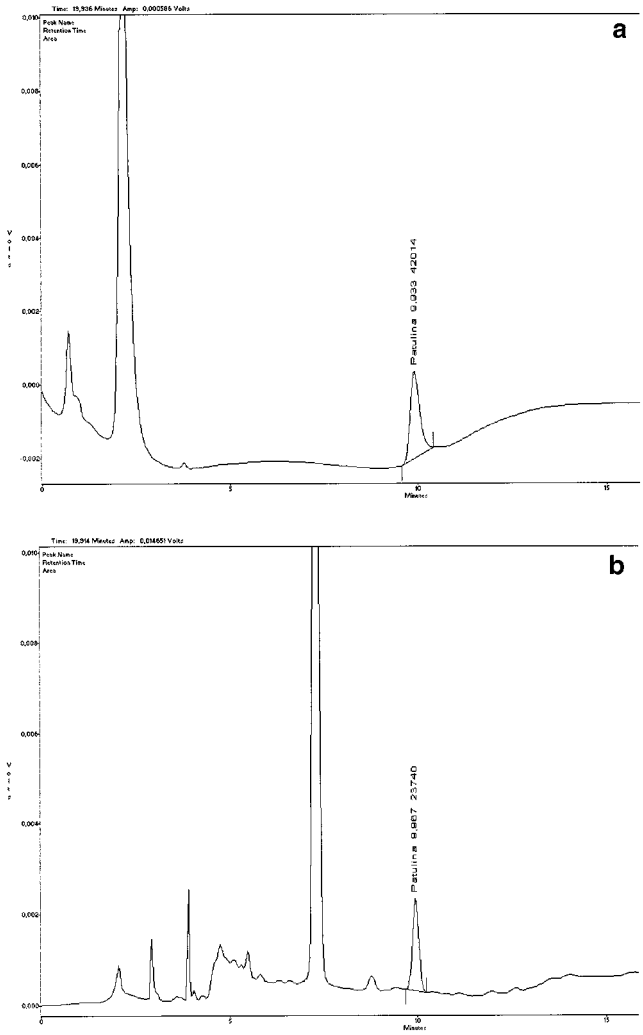

Figure 2. (a) Chromatographic run of pure patulin standard at the 50 μg/mL level. (b) Chromatographic run of positive sample T13.

Method C. According to procedure described by MacDonald et al. (*29*) with slight modifications, 10 g of apple products was diluted with 10 mL of pure water and 10 g of ammoniun sulfate and mixed for 15 min. This solution was centrifuged at 4000 rpm for 5 min at 4 °C and then filtered on Whatman paper. The cleared solution was extracted three times with 20 mL of ethyl acetate, and at the organic phase, sodium sulfate was added dry, filtered, and completely dried under reduced pressure at 40 °C. The organic residue was suspended in 1 mL of acetic acid solution at pH 4, and then, 20 μL was injected onto an HPLC column for analysis.

Chromatographic Conditions. HPLC analyses were performed using LC-10AD pumps and a diode array detector from Shimadzu (Japan). A Luna (Phenomenex, U.S.A.) C_{18} (250 mm × 4.6 mm, 5 μm) column was used. HPLC conditions were set up using a constant flow at 1 mL/min and CH_3CN-H_2O (10:90 v/v) as the starting eluent system, and the UV detector was set up at 276 nm. The starting ratio was kept constant for 13 min and then linearly modified to 100% CH_3-CN in 5 min. After 3 min at a constant ratio, the pumps were taken back to starting conditions in 3 min. In **Figure 2a**, a chromatographic run of patulin working solution at a concentration of 50 μg/mL is shown, and in **Figure 2b**, a chromatographic run of contaminated sample T13 is shown.

All samples were filtered through a 0.22 μm syringe filter (Millipore) prior to injection (20 μL) onto the column. Mycotoxin identification was performed by comparing retention times and UV spectra of purified samples to pure standard. A further confirmation was performed coinjecting samples together with patulin standard.

Mycotoxin quantification was carried out by comparing peak areas of investigated samples to the calibration curve of authentic standards.

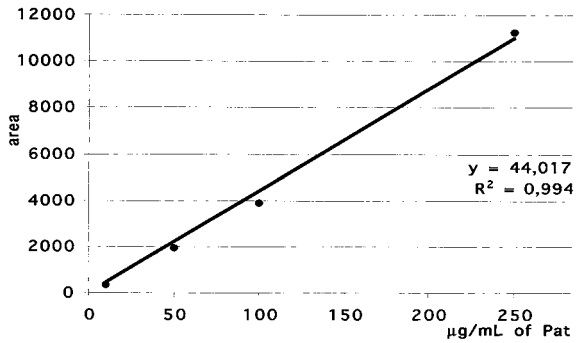

Figure 3. Calibration curve for patulin ranging from 10 to 250 μg/mL.

The retention time recorded for patulin in these conditions was 9.90 min, an average of 10 consecutive injections of the same patulin working solution.

The calculated instrumental detection limit and quantification limit for patulin were 0.1 (5 μg/L, S/N = 3) and 0.2 ng (10 μg/L), respectively. The UV spectra taken by the diode array detector during peak elution show that the patulin peak was free of any chromatographic interferences. In **Figure 3**, the calibration curve for patulin ranging from 10 to 250 μg/L and the related equation are shown. All results were statistically examined, and the relative standard deviation (SD) was calculated.

Recovery Experiments. To determine recovery efficiency for each protocol tested, a blank clarified apple juice product representative of juice, clarified juice, vinegar, and diluted puree was spiked at two levels (100 and 10 μg/L) with a patulin working solution. In addition, mousse for the mousse and baby food categories was homogenized in a Waring blender for 3 min with patulin spiking solutions before the extraction step. One hundred grams of clarified juice and mousse was spiked with 1 mL and 100 μL, respectively, of patulin working solution. Each test was performed three times, and the value shown is the average of three measures. All of the residue data shown were corrected for recovery.

RESULTS AND DISCUSSION

In the literature, several methods are used by the operator to extract and to analyze patulin from food sources such as juice, purees, or others. One of the most common extraction protocols requires ethyl acetate to extract patulin from juice and then washing of the organic solution with sodium carbonate to remove potentially interfering phenolic compounds (*30*, *31*). Other authors have slightly modified this method introducing solid phase extraction prepurification with silica before HPLC analysis (*32*). These procedures are fast and inexpensive, but on the other hand, they may destroy patulin, which is chemically a lactone, not stable at basic working pH. In addition, these protocols have some problems with no clarified juice or complex matrixes such as baby food or mousse. MacDonald et al. (*29*) suggest using an enzymatic approach. Pectinase avoids pectines from juice before HPLC analysis, and the digested solution is more easy to analyze because a large part of interferences is eliminated from solution. This extraction and prepurification procedure requires at least 2 h at 40 °C or incubation overnight at Ta.

This protocol has an economic cost for enzyme and requires a long time to obtain a partial clarification of sample juice. For these reasons, this method does not appear useful for large sampling and routine surveillance of apple products. Malone et al. (*27*) suggested prepurifying the samples through Mycosep columns that retained interfering impurities, and a good recovery (82−96%) was obtained. Several extraction methods were reported in the literature with good recoveries, using silica gel, florisil, Celite columns as prepurifying columns; a general limit of quantitation is considered 10 μg/mL (*28*).

Table 2. Recoveries Resume Obtained from Three Different Methods Applied

extraction method	spiking level (μg/kg)	average recovery calculated (%)	SD
	clarified juice		
A	100	31.4	1.55
A	10	24.0	1.21
B	100	63.5	1.70
B	10	69.0	2.14
C	100	79.2	0.49
C	10	82.5	6.06
	mousse		
A	100	28.4	1.18
A	10	25.2	2.02
B	100	58.6	1.92
B	10	54.3	2.25
C	100	74.2	2.12
C	10	73.8	4.45

Methods A−C above-described are three different approaches to optimize the extraction and prepurification procedure to analyze patulin. Patulin recoveries obtained with different methods described in the Material and Methods section are possible to divide in two macrocategories: first, juice, clarified juice, diluted purees, and vinegar; and second, mousse and baby food.

All of the results for clarified juice and mousse data are briefly summarized in **Table 2**. From these data, it is possible to obtain some information. Method A does not have a good recovery at high and low spiking levels (31.4 and 24.0%, respectively) probably due to protein parts of the juice that act as an adsorbent matrix for patulin reducing its recovery. In method B, both of the recoveries observed have been improved (63.5 and 69.0%, respectively) with respect to method A.

In this method, considered a slight modification of Gokmen et al. (*31*), no sodium carbonate was added during the extraction step to preserve the patulin occurrence. This method is useful for clarified juice; the interferences coextracted with patulin from unclarified juice reduce the method's performances.

Method C overcomes a large part of these problems and ensures a good recovery at low (82.5%) and high (79.2%) spiking tested levels. Protein components have been avoided from solution using ammonium sulfate without enzyme addition, and the cleared solution can be analyzed by HPLC without problems.

This method is not expensive and is very fast at analyzing several clarified apple products or unclarified apple products such as baby foods, mousse, or purees. To test efficiency and adaptability to different kinds of products in this method, it was applied to 40 commercial apple samples.

The results of the natural occurrence of patulin are reported in **Table 3**. Eleven samples, 27.5%, were positive for patulin analysis, and only sample T36, industrial puree to prepare commercial juice, was out of the suggested limit of 50 μg/mL. The range of patulin contamination is 1.4−74.2 μg/mL, and the average is 26.7 μg/mL (median, 17.70 μg/mL). All vinegar samples are negative for patulin, and this result is in accordance with Stinson et al. (*33*) because the enzymatic pattern of acetic fermentation of *Saccharomyces cerevisiae* downgrades patulin and hampers the development of *P. expansum*. Patulin was found in 20% of baby food up to the suggested limit of 10 μg/mL for this kind of food (samples T23 and T25). This is important evidence; patulin can be considered a typical mycotoxin with baby and children as the target, and for these subpopulations, the daily intake of apple juice or similar products

Table 3. Natural Occurrence Data for Each Apple Sample Analyzed

sample ID	agricultural practice claimed	comments	patulin (μg/kg)
T1	conventional	apple 100%	29.0
T2	conventional	apple 100%	<LD
T3	conventional	supermarket	5.8
T4	conventional	green apple juice	<LD
T5	conventional	retailer	56.4
T6	conventional	supermarket	<LD
T7	GMO	supermarket	<LD
T8	conventional	apple and other fruits	<LD
T9	conventional	apple and other fruits	<LD
T10	conventional	apple and other fruits	<LD
T11	GMO	apple and other fruits	<LD
T12	organic	supermarket	30.4
T13	organic	herbalist shop	33.2
T14	organic	herbalist shop	<LD
T15	organic	herbalist shop	1.4
T16	organic	herbalist shop	<LD
T17	organic	herbalist shop	<LD
T18	organic	herbalist shop	<LD
T19	integrated	supermarket	<LD
T20	organic	herbalist shop and apple with other fruits	<LD
T21	organic	herbalist shop and apple with other fruits	<LD
T22	conventional	baby food apple 100%	<LD
T23	organic	baby food from herbalist	17.7
T24	organic	baby food with banana from herbalist	<LD
T25	organic	baby food with strawberry from herbalist	13.1
T26	organic	baby food with peach from herbalist	<LD
T27	conventional	baby food	<LD
T28	conventional	baby food	<LD
T29	conventional	baby food with pear	<LD
T30	conventional	baby food	<LD
T31	conventional	baby food	<LD
T32	conventional	vinegar	<LD
T33	organic	vinegar	<LD
T34	organic	vinegar	<LD
T35	conventional	puree with antifermentative	16.7
T36	organic	puree with antifermentative	74.2
T37	integrated	puree with antifermentative	<LD
T38	conventional	puree pasteurized	15.9
T39	organic	puree pasteurized	<LD
T40	integrated	puree pasteurized	<LD

is very important considering their body weight. In fact, several countries regulate patulin at levels ranging from 20 to 50 μg/mL in fruit juices (*5*) and suggest reducing the limit of patulin for baby and children food to 20 μg/mL.

Considering agricultural practices claimed on food labels, no significant differences were found between organic and conventional products. Conventional products show patulin levels ranging from 5.80 to 56.40 μg/mL with an average of 24.76 μg/mL, while organic products range from 1.40 to 74.20 μg/mL with an average 28.34 μg/mL. Finally, industrial purees ready to dilute to commercial juice give interesting results. A higher contamination has been found in organic sample T36 added with antifermentative (sodium metabisulfythe). The layout process of this sample requires one pasteurization step of the purees after addition of antifermentative. On the contrary, samples without the addition of antifermentative require two thermal treatments. Purees treated in this way are more safe with respect to samples with the addition of antifermentative and only one pasteurization step.

Comparing samples T38 and T35, patulin levels are comparable (15.90 and 16.70 μg/L, respectively); consequently, antifermentative addition to determine contamination level is not but fungi occurrence is comparable. We obtained an interesting result with regard to the sample claimed "integrated" on the labels. Unluckily, only three products are claimed integrated. Anyway, all of these products are negative for patulin, and they are considered a good compromise between organic and conventional approaches.

Method C appears to be easy, fast, and economic, and its validation on a large apple product sampling has been performed. Method C ensures a good recovery from different kinds of food matrixes, and it permits a fast routine analysis. Finally, the situation of natural occurrence of patulin in Italian marketed drink and food appears to be good, according to Beretta et al. (*34*), especially if compared with English surveillance (70% positive samples; *35*) and Australian surveillance (50% positive samples; *4*).

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