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Study of thermal resistance and *in vitro* bioaccessibility of patulin from artificially contaminated apple products

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This paper is dedicated to the memory of Prof. Maria Malanga

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ABSTRACT

Apple juices and purees represent categories widely consumed by whole population and above all children. Patulin (PAT) is a mycotoxin known for its acute and chronic effects in animals. Several studies indicate there is a risk associated to the PAT intake, through the consumption of purees and apple juices. In this study, apple juice and puree were prepared and artificially contaminated with PAT at 50 μg/kg and submitted to a thermal treatment simulating pasteurization to evaluate PAT's reduction. In a second phase of the work, apple products samples ($n = 7$) included juices, nectars and purees belonging to different commercial brands were collected, artificially contaminated with PAT at 50 μg/L (limit established for PAT in juices) and 25 μg/kg (limit established for PAT in purees), digested with an *in vitro* gastrointestinal protocol and bioaccessibility values (%) were calculated.

After thermal treatment, the PAT's loss evidenced in purees and juices was of 1.41 ± 0.52% and 62.62 ± 2.53% respectively. Related to the bioaccessibility data, two juices with pulp showed values of 70.89 ± 4.93 and 67.30 ± 10.76%; two purees showed levels of 58.15 ± 5.50 and 55.69 ± 4.73%, whereas nectar and two clarified juice showed percentages of 38.88 ± 2.42, 28.59 ± 0.46 and 25.28 ± 0.61%, respectively.

1. Introduction

Patulin (PAT) is a toxic secondary metabolite produced by a number of fungal species belonging to the genera *Penicillim*, *Aspergillus*, and *Byssochlamis*. Particularly *Penicillium expansum* is known as the main source of PAT and is commonly associated with apple rot (Desmarchelier et al., 2011).

This mycotoxin was initially identified as an antibiotic but it is also toxic towards plants and animals, evidencing a Median Lethal Dose (LD_{50}) in mice of 5 mg/kg (Trucksess and Tang, 1999). Although PAT can occur in many mouldy fruits, grains and other foods, the major sources of contamination is represented by apples and apple products. Acute symptoms of PAT exposure include agitation, convulsions, edema, intestinal inflammation, vomiting and ulceration; chronic effects include genotoxicity, immunotoxicity, and neurotoxicity in rodents, while its effects on humans have not been demonstrated yet (Speijers, 2004). Dickens and Jones (1961) found a significant increase in tumor incidence in animals treated with PAT in comparison with controls. The International Agency for Research on Cancer (IARC) evaluated the toxicity data and classified PAT in Group 3 carcinogen or "a compound for which there is not enough data to allow its classification" (IARC, 1986).

Consumption of fruit juice helps fulfil the dietary recommendation to eat more fruits and vegetables, with fruit juice accounting for 50% of all fruit servings consumed by children aged two through 18 years, and 1/3 of all fruits and vegetables consumed by preschoolers. According to fruit products manufacturers, children under 5 years of age consume, on average, from 90 mL/day of fruit juice until to 170 mL/day (Dennison et al., 1996). Moreover, 11% of healthy preschoolers consumed ⩾340 mL/day of fruit juice (Barbara and Dennison, 1996). In the European Union, fruit juices and nectars consumption varies considerably among member states depending on climatic conditions and habits of consumption, and amounted, in average, to 110 mL/day in 2007 (Maja, 2010).

Since 2006, an European Commission Regulation (CE 1881/2006) establishes a maximum limit of 50 μg PAT/kg for fruit juices, reconstituted concentrated fruit juices, fruit nectars, spirit drinks, ciders and other fermented drinks derived from apples or containing apple juice and 25 μg PAT/kg for solid apple products, including apples compote and apples puree for direct consumption. As regards the children, considering that they represent a vulnerable population for their physiology and lower capacity to metabolize toxic compounds, and due to the high consumption of apple products during the first years of life, are more exposed to PAT toxicity

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compared to adults (Moake et al., 2005). Therefore, a limit of 10 μg/kg was fixed for PAT in apple juice, solid apple products, including apple compotes and apple purees baby-foods and cereal-based-foods, for infants and young children.

Several studies have been carried out in different countries on PAT presence in apple and its products. Studies carried out in Italy reported mean PAT levels of 24.8 μg/kg (conventional apple juices), and 28.3 μg/kg (organic apple juices) (Ritieni, 2003), and 3.1 and 7.1 μg/kg from conventional and organic apple juices, respectively (Piemontese et al., 2005). More recently, Barreira et al. (2010) studied the occurrence of PAT in apple-based foods in Portugal, detecting that 23% of the samples had values between 1.2 and 42 μg/kg. In summary, these data indicate that nowadays there is a risk associated to the PAT intake through the consumption of apple-based foods.

In human health risk assessment, ingestion of food is considered a major route of exposure to many contaminants either caused by industrial or environmental contamination or as result of production processes, although the total amount of an ingested contaminant (intake) does not always reflect the amount that is available to the body. In this sense, bioaccessibility has been defined as the fraction of a compound that is released from its matrix in the gastrointestinal tract and thus becomes available for intestinal absorption (Benito and Miller, 1998). The bioaccessibility of several mycotoxins has been studied by authors like Avantaggiato et al. (2003, 2004), which have assessed the intestinal absorption of zearalenone (ZEA), fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), ochratoxin A (OTA), deoxynivalenol (DON) and aflatoxin B_1 (AFB_1).

The content of patulin decreases by only 25% during the heating processes of fruit juice production (Kadakal and Nas, 2003).

Therefore, the aims of this work were: (a) to evaluate the PAT thermal degradation in apple products during a thermal treatment; (b) to study PAT's bioaccessibility in apple products through an *in vitro* digestion model which reproduces the physiological conditions of the children.

This study can represent an original contribution to the mycotoxin risk assessment because, for the first time, evaluates the processing conditions and the bioaccessibility of PAT in fruit products widely consumed by children.

2. Materials and methods

2.1. Reagents

Potassium chloride (KCl), potassium thiocyanate (KSCN), monosodium phosphate (NaH_2PO_4), sodium sulphate ($NaSO_4$), sodium chloride (NaCl), sodium bicarbonate ($NaHCO_3$), urea, α-amilase, hydrochloric acid (HCl), pepsin, pancreatin, bile salts were obtained from Sigma–Aldrich (Madrid, Spain). Ethyl acetate, hexane, acetonitrile, methanol, water, acetic acid were purchased from Merck (Milan, Italy). Deionized water (<18 MΩ cm resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Chromatographic solvents and water were degassed for 20 min using a Branson 5200 (Branson Ultrasonic Corp., CT, USA) ultrasonic bath. PAT standard was purchased from Sigma–Aldrich (Milan, Italy).

2.2. Preparation of working standard solutions

One milligram of PAT standard (4-hydroxy-4H-furo[3,2-c]pyran-2(6H)-one, Sigma Chemicals, St. Louis, MO) was weighed and diluted to 10 mL with methanol (Merck, Darmstadt-Germany) to obtain a stock solution of 100 mg/L. The working standard solutions were obtained by dilutions of the stock solution. The solutions were stored in the dark at 4 °C before using.

2.3. Sampling

Fresh apples were acquired in September 2011 from different markets located in Naples (Italy), belonged to Golden Delicious variety. These samples were employed for evaluating PAT degradation due to the thermal treatment, as well as to study PAT's bioaccessibility by using an *in vitro* digestion model.

Totally seven samples of commercial products were studied and divided in: two clarified apple juices, two apple juices containing pulp, one apple and pear nectar, two apple purees. These samples belonged to different brands and were collected from different supermarkets located in Naples (Italy) in October 2011 and were used for assessing PAT's bioaccessibility.

2.4. Thermal treatment tests

One kilogram of apples was homogenized using a blender (Bosch 600 W; Milan, Italy) at 10,000 rpm during 3 min, and centrifuged at 15,000 rpm during 3 min to separate the juice from the solid fraction.

Twenty-five grams of juice and puree were artificially contaminated with PAT at levels of 50 μg/L and 50 μg/kg, respectively. Then, the apple products were treated with a process constituted by three steps simulating pasteurization: (a) first pasteurization at 80 °C during 20 min; (b) cooling at 4 °C in 30 min; (c) second pasteurization at 80 °C during 20 min. Each product (juice and puree) was treated in triplicate and a control test was also carried out (product not thermally treated).

2.5. PAT extraction

For PAT extraction, the method of Arranz et al. (2005) was applied with slight modifications.

In particular, 10 g of sample were transferred into a 50 mL-plastic tube containing 15 g of Na_2SO_4, 2 g of $NaHCO_3$ and 10 mL of extraction solution (ethyl acetate/hexane 60:40, v/v). The tube was shaken for 3.5 min on a mechanical shaker (Intercontinental equipment, Hidalgo, TX) and then centrifuged at 2000 rpm for 1 min at 4 °C. The supernatant (2.5 mL) was immediately placed onto an unconditioned Strata C18-E solid phase extraction column (Phenomenex, USA) which was then washed with 3 mL of the extraction solution adjusting the flow to 1 drop per second by using slight air pressure. The eluate was collected in a 10 mL-plastic test tube containing 50 μL of acetic acid. The solvent was evaporated at max 40° C for about 35 min in a vacuum centrifuge Thermo Savant (Speed Vacuum Thermo Electron Corporation Milford, MA, USA) and 1 mL of acidulated water (pH 4) was used to dissolve the sample. The solution was mixed with a vortex (Biosan MSV-3500, Lietsa, Finland) filtered with 0.22 μm filters (Phenomenex, Palo Alto, CA) and injected in the LC apparatus.

2.6. PAT LC analysis

PAT determination was carried out according to Arranz et al. (2005) by using an LC apparatus (Shimadzu-Japan) equipped with an autosampler SIL-20A, two pumps LC-20AD and a UV/vis detector SPD-20A set at 276 nm wavelength. The column was a Gemini 5 μ C18, 110 Å (150 mm × 2 mm) (Phenomenex, CA, USA). The mobile phases were water containing 1% of acetic acid (A) and methanol (B) in isocratic conditions (95/5, v/v). The flow rate was of 1 mL/min. Under these chromatographic conditions, the retention time for PAT was 14.0 ± 0.1 min.

2.7. In vitro digestion model

Commercial samples, previously checked not to be contaminated with PAT, were artificially contaminated at 50 μg PAT/L (legislative limit fixed for PAT in juices) for juices and at 25 μg PAT/kg (legislative limit fixed for PAT in solid products) for purees, to carry out the bioaccessibility tests simulating gastrointestinal conditions of children, characterized by lower levels of pancreatin, pepsin and bile salts respect to adults.

The procedure of the *in vitro* digestion was adapted from the method described by Gil-Izquierdo et al. (2002) and Jovaní et al. (2004). The method consists of two sequential steps: an initial saliva/pepsin/HCl digestion for 2 h at 37 °C, to simulate the mouth and the gastric conditions, followed by a digestion with pancreatic juice for 2 h at 37 °C to simulate duodenal digestion.

For the saliva/pepsin/HCl digestion, 10 g of sample were mixed with 80 mL of distilled water and 6 mL of artificial saliva composed by: KCl (89.6 g/L), KSCN (20 g/L), NaH_2PO_4 (88.8 g/L), $NaSO_4$ (57 g/L), NaCl (175.3 g/L), $NaHCO_3$ (84.7 g/L), urea (25 g/L) and 290 mg of α-amilase. The pH of the solution was adjusted at 6.8 with HCl 0.1 N. Immediately after, 0.02 g of pepsin dissolved in HCl 0.1 N was added to the sample, the pH was adjusted at 3 with HCl 6 N, and then incubated at 37 °C in an orbital shaker (250 rpm) (Infors AG CH-4103, Bottmingen, Switzerland) for 2 h.

After the gastric digestion, the pancreatic digestion was simulated. The pH was increased to 6.5 with $NaHCO_3$ 0.5 N, 0.0005 g of pancreatin and 0.03 g of bile salts dissolved in 20 mL of water were added and incubated at 37 °C in an orbital shaker (250 rpm) for 2 h. After the pancreatic digestion, 30 mL of the mixture were centrifuged at 4000 rpm and 4 °C during 1 h. To determine the PAT bioaccessibility from the different samples, 10 mL of the intestinal phase (pancreatin–bile digestion) were analyzed according to the method described in the Sections 2.5 and 2.6 and it was expressed as percentage of bioaccessibility.

2.8. Statistical analysis

All tests were conducted in triplicate. Data of PAT bioaccessibility and thermal stability were subjected to analysis of variance (ANOVA). Duncan Multiple Range Test was used to evaluate the mean values of bioaccessibility in different categories and it was determined a significance level of 5%.

3. Results

3.1. Analytical performance

For PAT recovery studies, 10 mL (or 10 g) of uncontaminated apple juice (or puree) were spiked at levels of 10, 50 and 100 μg/L of PAT with the standard dissolved in methanol. Biological fluids obtained from digestion of uncontaminated apple products were also spiked with PAT at the same concentrations to develop recovery tests. For each level of contamination the test was performed in triplicate. The samples and digested fluids stood overnight at room temperature and then extracted according to the method explained in Section 2.5.

The mean PAT recoveries were independent of the spiking levels assayed and ranged from 78.1 ± 5.4% in puree (digested and not digested) to 96.4 ± 6.3% in juice (digested and not digested), respectively, with relative standard deviations (RSD) below 15%. The limit of detection (LOD) of the PAT method was 5 μg/kg, while limit of quantification (LOQ) was 15 μg/kg. PAT quantification was carried out by including signal area values into a linear calibration curve performed with PAT standards, within the range of 5–100 μg/L and by correcting errors imputable to variable sensitivity of instrument intra-day due to minimal intra-day instability.

3.2. PAT degradation during thermal treatment

The effect of the thermal treatment on PAT reduction in apple juice and puree artificially contaminated (50 μg/L and 50 μg/g, respectively) is shown in chromatograms (Fig. 1a and b) and in Fig. 2. In purees samples, results did not show a significant reduction according to Duncan's test (p value = 0.05) whereas in the juices the reduction was statistically significative, (p value = 0.05), equal to 62.62 ± 2.53% (from 50 to 19.10 μg/L). The lowest efficacy of the thermal treatment on PAT degradation in puree could explain the major incidence of the mycotoxin in apple puree respect to juice as reported in literature.

3.3. PAT bioaccessibility

Bioaccessibility percentages of PAT in seven selected artificially contaminated commercial apple products after the *in vitro* digestion process are shown in Table 1. The samples that showed the significantly (p value = 0.05) highest percentage of bioaccessibility were apple juices with pulp (70.89 ± 4.93% and 67.30 ± 10.76%), followed by the puree samples (58.15 ± 5.50% and 55.69 ± 4.73%). The categories characterized by the lowest, and significant (p value = 0.05) different among them, levels of bioaccessibility were apple nectar and the clarified juices, with 38.88 ± 2.42%, 28.59 ± 0.46% and 25.28 ± 0.61%, respectively.

Mean bioaccessibility values from clarified juices and containing-pulp juices and purees were 30.92 ± 7.09% and 63.01 ± 7.25%, respectively. The mean data for these different categories resulted significantly different (p value = 0.05). This aspect suggested that the differences in the matrixes have influenced on the mobilization of the mycotoxin from the food. In this sense, it seems that the physical structure of the clarified products could have favoured

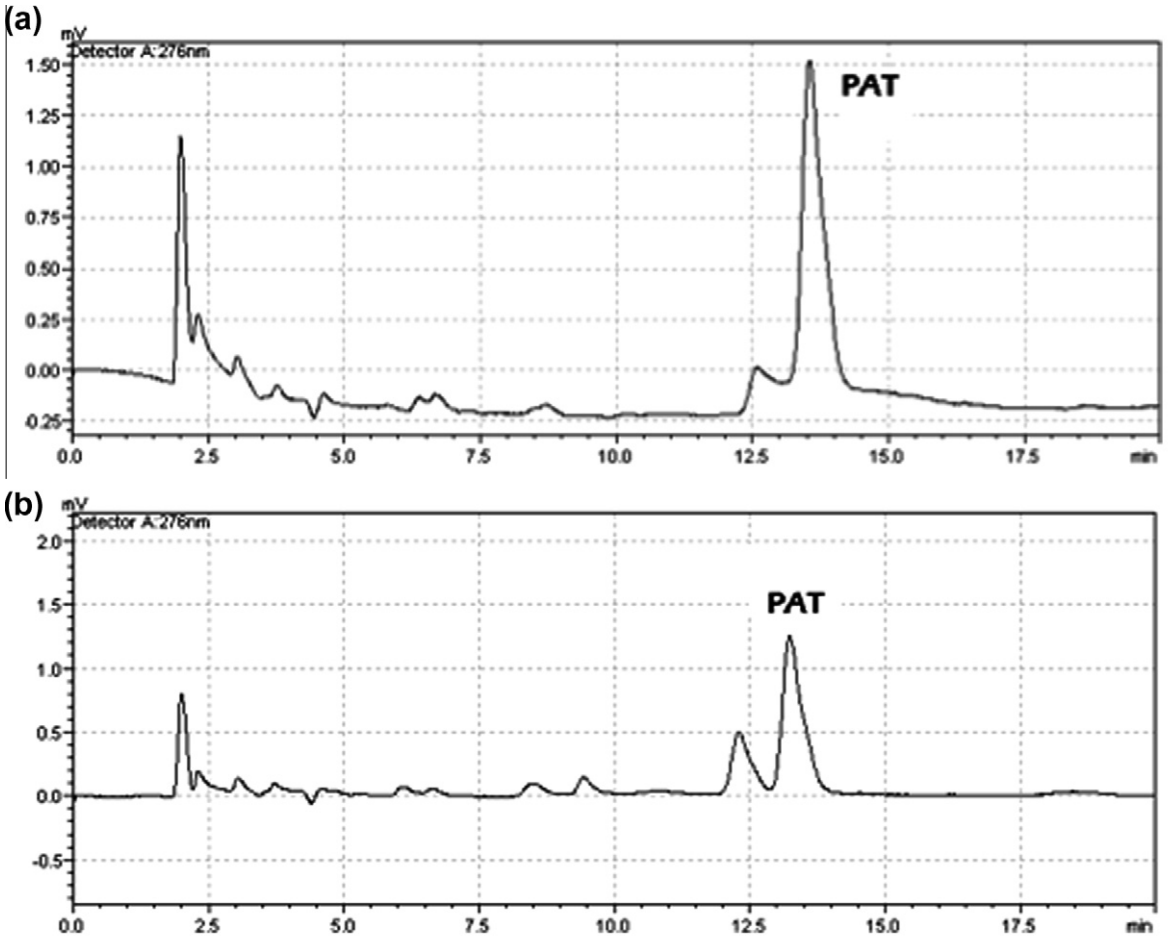

Fig. 1. Ultraviolet chromatograms of PAT in: (a) apple mousse artificially contaminated at 50 μg/kg before heat treatment. (b) apple mousse artificially contaminated at 50 μg/kg after heat treatment.

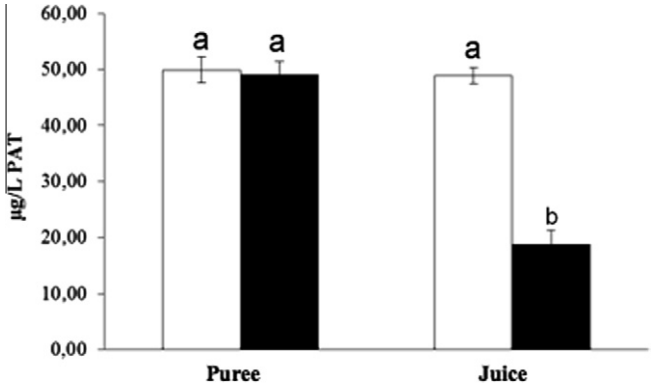

Fig. 2. Thermal degradation of PAT in apple puree and juice artificially contaminated at 50 µg/L. White columns = pre heat treatment and black columns = post heat treatment.

Table 1
PAT bioaccessibility *in vitro* in seven selected apple products.

Food product	% Bioaccessibility
Apple juice with pulp	$70.89^{b} \pm 4.93$
Apple juice with pulp	$67.31^{bd} \pm 10.76$
Apple puree	$58.15^{cd} \pm 5.50$
Apple puree	$55.70^{bc} \pm 4.73$
Apple and pear nectar	$38.88^{a} \pm 2.42$
Apple clarified juice	$28.60^{e} \pm 0.46$
Apple clarified juice	$25.28^{e} \pm 0.61$

Mean values with the same letter are not significantly different (p value = 0.05) (Duncan's test).

the action of digestive enzyme, while in the presence of fruit pulp, this effect could be disadvantaged.

Generally, absorption and metabolism depend more on the compound specific properties and physiology and, therefore, the matrix is expected to have less influence on these processes. However, in some cases, the ingested matrix has been shown to affect the transport of the contaminant across the intestinal epithelium (Wienk et al., 1999; Brandon et al., 2006).

Considering the PAT initial concentration and their bioaccessibility percentages, it can be calculated that 15.5 µg PAT/L and 24.4 µg PAT/kg in clarified juices and pulp-containing products (juices with pulp and purees), respectively, could interact, with the intestinal epithelium cells in children over 3 years old. These results show that lower limits of regulation for children are needed, as juices at the regulatory level for adults are still considerably contaminated after any heating and after passing the gastrointestinal tract. An insufficient knowledge on the bioaccessibility may hamper an accurate risk assessment of ingested contaminants in humans.

4. Discussion

Industrial processes such as depectinization, clarification and filtration applied in juice production can reduce the levels of PAT between 20.5% and 39.1% (Acar et al., 1998; Bissessur et al., 2001). In this sense, Funes and Resnik (2009) determined PAT in solid and semisolid apple and pear products marketed in Argentina. Results showed that 21.6% of products were contaminated (mean of positive samples was of 61.7 µg/kg), and the highest levels were found in apple purees with 50% of the contaminated samples (mean of positive samples of 123 µg/kg).

Several studies have been carried out on PAT reduction during industrial manufacturing. Taniwaki et al. (1989) treated thermally (90 °C for 2 min) an apple juice contaminated with PAT (1500 µg/L), followed by hot-filling and a final 5 min heat treatment in boiling water followed by a cooling at room temperature. Results showed a 60% of PAT reduction, similar to our values. Lower reduction percentages were found after thermal treatments of 90 °C for 10 s (Kadakal et al., 2002) and 90 °C and 100 °C for 20 min (Kadakal and Nas, 2003) of apple juices, obtaining 13.4%, 18.81%, and 25.99% of mean reduction of the bioactive compound, respectively.

Nowadays, few studies on bioaccessibility of mycotoxins have been carried out. Versantvoort et al. (2005) evaluated the bioaccessibility of AFB_1 from peanut slurry and OTA from buckwheat. Both mycotoxins were almost completely mobilized from the peanut slurries during digestion, at levels of 94% and 100%, respectively. In another study on AFB_1 bioaccessibility from grinded corn and peanut by means of an *in vitro* digestion experiment (Simla et al., 2009) values of 95% and 94%, respectively, were reported. More recently, Dall'Asta et al. (2009) applied an *in vitro* digestion model to raw maize in order to evaluate the possible release of hidden fumonisins under gastrointestinal conditions. After digestion, an increased amount of total detectable fumonisins was observed in comparison with the analysis on the non-digested matrix.

In conclusion, data found in this study showed that the applied thermal treatment produced a PAT reduction, close to those found in literature for apple juice samples. Moreover, bioaccessibility results showed that, in the worst case scenario (for samples contaminated at maximum allowed PAT levels), deleterious levels of the mycotoxin could interact with the intestinal epithelium cells, and it should be considered for the risk assessment in children who represent a category that usually consume fruit juices and purees.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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