JOURNAL of Horticulture, Forestry and Biotechnology
Volume 28(2), 2024, 220-226
https://jhfb.ro/index.php/jhfb
Print ISSN: 2066-1797
Online ISSN: 3045-1876

A review of patulin, "the mycotoxin of apples", and its methods of detection

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Manuscript received: 8 November 2024; revised: 14 November 2024; accepted: 15 November 2024

Abstract
As a consequence of their development on food and feed, filamentous fungi are able to synthesize, as secondary metabolites, toxic elements with low molecular weight, named mycotoxins. The mycotoxin named patulin is synthesized by different fungal species, in different fruits, vegetables, cereals or nuts, but it is characteristic to apple fruits and derived products. Among the fungal species, the main patulin producer in apples is *Penicillium expansum* Link, which causes the blue mold (soft rot) in the fruits during storage period. With *P. expansum* contamination come several risks regarding financial losses for the farmers and the health of the consumers, in case of mycotoxin-contaminated product ingestion. Because of its chemical stability, heat-resistance and water-solubility, patulin can't be destroyed completely by processing. The biggest worry about patulin is its possible presence not only in fruit juices and purees, but in baby food too. Taking into consideration the great number of negative effects patulin ingestion has on human health (immunodepressant, neurotoxic, teratogenic, gastrointestinal side effects and others), the EU needed to establish maximum tolerable levels in juices and other derivatives. As set by the European regulation 1425/2003, the highest accepted patulin level in apple juice is 50 µg/l, 25 µg/l in solid apple products and 10 µg/l in apple-based baby foods. For manufacturers and authorities, analytical procedures for patulin determination are important tools, in order to be able to provide high levels of food and feed safety. In the detection and quantification of patulin, various chromatographic techniques dominate, but alternatives, such as immunological assays and biosensors, start to gain more and more attention. This paper aims to provide a general overview of patulin and a brief description of the various methods used for its identification in apple-based products.

Keywords: patulin, *Penicillium expansum*, blue mold, apple

Introduction

Apples are one of the most consumed fruits worldwide, with a global apple production of 83.74 million metric tons in 2023, according to USDA (U.S. Department of Agriculture) Foreign Agricultural Service) [46]. According to FAOSTAT (Food and Agriculture Organization of the United Nations), in 2022 the global apple production was 95835964.97 t, harvested from an estimated area of 4825729 ha [48].

Filamentous fungi are found everywhere in nature and these microorganisms frequently attack food crops both on the field and during post-harvest storage. As a result of their high adaptability to their surroundings [14], they are able to survive within a variety of environmental conditions regarding pH, temperature or humidity [7]. As a consequence of their development on food and feed, filamentous fungi are able to synthesize, as secondary metabolites, toxic elements with low molecular weight, named mycotoxins [7, 16].

So far, about 400 mycotoxins have been identified, but only a limited number are known to be dangerous for humans and animals, for instance aflatoxins, fumonisins, zearaenone, patulin and ochratoxin A [2, 9, 18].

The main mycotoxin-producer fungi are *Aspergillus* spp., *Penicillium* spp., *Alternaria* spp. and *Fusarium* spp. However, fungal presence does not automatically mean upcoming mycotoxin infection,

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Volume 28(2), 2024, 220-226
https://jhfb.ro/index.php/jhfb
Print ISSN: 2066-1797
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as mycotoxins require specific conditions in order to be produced, separate from those that contribute to the growth of the fungi [7].

Main producers of patulin

The mycotoxin named patulin (4-hydroxy-4H-furo[3,2-c]pyran-2(6H)-one) is a heterocyclic lactone with low volatility, melting point at 110℃, and stability at 105-125℃ in aqueous media [26]. It is a secondary metabolite synthesized by different fungal species, predominantly belonging to *Penicillium, Aspergillus* and *Byssochlamys* genera. Amongst *Penicillium* species, thirteen are known to produce patulin (namely *P. expansum, P. clavigerum, P. griseofulvum, P. carneum* and others), but some *Aspergillus* species can also produce this mycotoxin (for example *A. clavatus* and *A. giganteus*). From *Byssochlamys* genera, only *B. nivea* can produce patulin [16]. According to Sant'Ana et al. (2008) [36] and Cunha et al. (2014) [6], *Aspergillus* species are responsible for patulin secretion in warm and humid climates, such as tropical regions, while *Penicillium* species produce the toxin in colder environments.

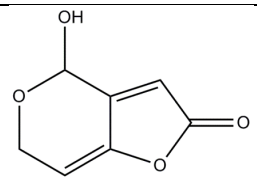

Figure 1. Chemical structure of patulin
Source:
(https://www.researchgate.net/publication/316866233_Mitigation_of_Patulin_in_Fresh_and_Processed_Foods_and_Beverages)

Patulin is present in many different fruits, vegetables, cereals and nuts, as well as their derived products, but mostly in apples and its derivatives (juice, cider, compote, puree) [13,21]. Among the fungal species, the main patulin producer in apples is *Penicillium expansum* Link, which causes the blue mold (soft rot) in the fruits during storage period [2,47].

With *P. expansum* contamination come several risks regarding financial losses for the farmers and the health of the consumers, in case of mycotoxin-contaminated product ingestion [19,23]. For these reasons, this topic needs to be taken very seriously.

The fungus enters the fruit where the conidia germinate and begin to spread starting from that point. Rotting starts to develop and grow rapidly. Maximum concentrations of patulin in fruits are commonly found within 1 cm of the damaged area, but it has been demonstrated that the toxin can migrate within the fruit [24]. At the penetration site of the fungus, the central part of the spoiling area, a whitish-coloured mold appears, that will later develop greenish-blue conidia and conidiophores [42]. Wind, insects and water transfer the conidia from one fruit to another, which can lead to further contaminations. There are exceptions, when the fungus penetrates through the stalk. In cases like this, the rot develops inside the fruit, and no visible symptoms are found on the surface of it. If these fruits are not eliminated prior to processing, they can be a source of patulin contamination [26].

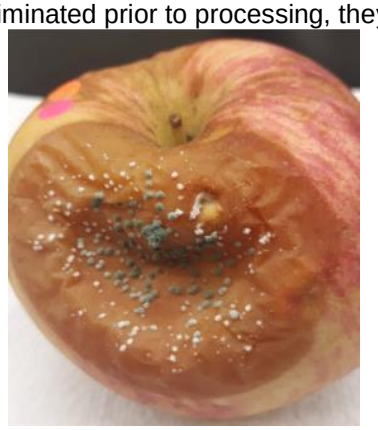

Figure 2. Blue mold on apple (watery, soft lesion, with white and green spores) (Source: https://treefruit.wsu.edu/crop-protection/disease-management/blue-mold/)

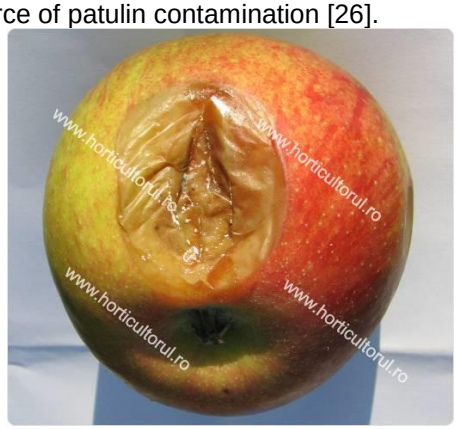

Figure 3. Soft rot (blue mold) (Source: https://www.horticultorul.ro/insecte-boli-daunatori-fungicide-insecticide-ingrasaminte-pesticide/putregaiul-albastru/)

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Influencing factors

Bruised fruits are more susceptible for being infected with the fungus. Damages, including mechanical injuries, cuts, insect or birds wounds, crackings of the fruits, can occur both in the field (for example due to unfavorable weather conditions) and during harvesting, delivering, packaging or even during postharvest storage [19,20]. Although getting rid of rotten parts of the fruits can reduce the patulin degree in juices, the mycotoxin can't be completely eliminated, since it can migrate to healthy portions of the fruit [2].

Several environmental factors, internal factors, as well as the interactions between them have an impact on patulin production. These include temperature, fruit pH, water activity, humidity, oxygen concentration, geographical location, substrate, microbial load on the fruit, fungal strain, fruit variety, ripening degree, skin thickness, flesh firmness, sugar content and other physiological properties of the fruits [2, 16, 19, 25, 41].

Moulds, in general, grow at temperatures between 20-37℃, with an optimum of 25℃ for *P. expansum*, and mycotoxin production can happen at 25.5 ± 5.5℃. Secretion of patulin can take place at lower temperatures too (below 10℃), as *P. expansum* is able to survive even at 0℃, and below (-3℃); in this case, production period is longer and concentration of the toxin decreases with decrease of temperature [2, 16, 20, 26].

Patulin has high stability in acidic (pH range from 3.5 to 5.5) environment, but is easily degraded under basic conditions. pH from 4.0 to 5.0 stimulates *P. expansum* spore germination, while very acid pH such as 2.0 or alcaline pH (8.0) have an inhibitory effect on spore germination [20,21,26]. The effect of pH on patulin production by *P. expansum* was studied by Coton et al. (2020) on apples in cold and ambient storage, and they concluded that germination and growth of the spores take place in apples at a pH as low as 3-5 [5].

The optimal fluctuation of water activity is between 0.83 and 0.9 aw. High relative humidity (70–90%) and moisture content (20–25%) increase fungal growth and toxin formation as well [16, 20].

Regarding oxygen concentration, *P. expansum* can be found at as low as 2% O_2 concentration in the atmosphere [2].

Maturity grade of the fruits is a decisive parameter in predisposition to fungal infection during postharvest storage. A ripe fruit is characterized by high sugar content, pH changes, reduced firmness due to water loss and reduced defense systems [19].

Patulin production is also strongly affected by the genotype of the crop, as it impacts its ability for wound healing and vulnerability to infection [2, 23].

Patulin presence in apple derivatives

Because of its chemical stability, heat-resistance and water-solubility, patulin can't be destroyed completely by processing. Also, the death or inactivation of the fungus, do not guarantee that patulin will dissapear from the contaminated product [2,16,21,26,44].

The biggest worry about patulin is its possible presence not only in fruit juices and purees, but in baby food too. Taking into consideration the great number of negative effects patulin ingestion has on human health, the EU needed to establish maximum tolerable levels in juices and other derivatives [43]. As set by the European regulation 1425/2003, the highest accepted patulin level in apple juice is 50 µg/l, 25 µg/l in solid apple products (apple compote, apple puree) and 10 µg/l in apple-based baby foods (for infants and young children) [45].

As specified by the European Commission (EC) the actual maximum tolerable daily intake is 0.4 µg/kg body weight [26]. The Joint Expert Committee on food Additives of the World Health Organization (JECFA) also recommended this daily maximum limit [30].

Studies carried out in different countries such as Italy [28], Spain [29] or Belgium [3] have focused on the comparative analysis of patulin between conventional and organic products. Most of the studies show that contamination is significantly higher in organic products, but there are exceptions too, where there was no significant difference [6].

Effects on human health

The degree of a mycotoxins' adverse effects is determined by its chemical structure, the quantity of the toxin and the period of exposure to it [38], as well as by individual susceptibility of the food products [11].

Acute patulin exposure can be the cause of gastrointestinal disorders which consist of nausea, intestinal bleeding, vomiting, ulcers and duodenal injury [26, 27]; other symptoms may include convulsions, pulmonary congestion, restlessness, dyspnea and kidney damage [21].

Chronic exposure to patulin, and mycotoxins in general, could substantially modify the regular composition of intestinal microbiota [26], but genotoxicity, neural disorders and immunotoxicity are also proved negative effects [2, 15, 35, 36].

Exposure of humans to patulin might have other consequences as well, including long congestion [15,36], brain haemorrhage [10], indirect enzyme-inhibition, pulmonary or even cerebral edema, gastritis, capillary damage, seizures [31], allergic reactions [39]. The liver and kidneys are also predisposed to being affected by the mycotoxin [33].

Shi et al. (2019) concluded that even at low concentrations, these toxins manifest a broad range of bioactivities, which include teratogenic, cytotoxic and mutagenic effects [37], while Erdem & Senturk (2024) consider that at low levels, patulin exposure does not represent a typical risk factor [11].

Carcinogenic property of patulin has also been highly studied. Presently it is classified in Group 3 by the International Agency for Research on Cancer (I.A.R.C.), meaning that it is a possible carcinogenic substance, but since there is not enough evidence to prove this, it is not yet included in confirmed carcinogens [2,26].

Even though high patulin intake has a negative influence on all age groups, young children are especially under high risk, proven by the accepted patulin level in apple-based products for babies, which is five-times lower than in apple juice. Higher vulnerability of children is not only due to the higher exposure per kilogram of body weight, but also their physiological differences in comparison to that of adults [8, 32, 34].

Methods of detection

For manufacturers and authorities, analytical procedures for mycotoxin determination are important tools, in order to be able to provide high levels of food and feed safety [9].

There isn't an official method for patulin analysis, each research laboratory can implement its own methodology, but in all cases some specific parameters need to be respected. Repeatability and reproducibility are key parameters for the standardization of different methods [26].

Regardless the analytical method, the first step in mycotoxin detection is always sample collection, and afterwards sample preservation until analysis is done [25]. Homogenization is vital because mycotoxin distribution is heterogenous, and it is of great importance that samples are homogeneous.

The next step is the extraction of the toxin from the sample, which is effected differently for every type of mycotoxin, but the steps are the same, as follows: grinding, homogenization with solvents and filtration. Solvents used, up to now, in the extraction operation are water, methanol, acetone, chloroform, acetonitrile and potassium chloride [25].

Purification is mandatory after the extraction process, for the separation of the target molecules from other co-extracted substances that could interfere with the analyte throughout the detection process. The most recent and most effective purification method is utilizing immune-affinity column (IAC), which is specific for every mycotoxin, as it contains antibodies [25].

The selection of the instrument that does the analytical detection is the key factor for the favorable outcome of the analysis [25].

In the detection and quantification of patulin, various chromatographic techniques dominate. The first method used was thin layer chromatography-TLC, which is an affordable and simple technology, does qualitative and quantitative analysis of mycotonxins, but it is rather imprecise. The most popular method in analysis of patulin is high performance liquid chromatography- HPLC, that separates mycotoxins and determines their quantity based on their chemical attributes, used in combination with photodiode array or UV detectors. It can achieve a 5 mg/l detection limit approximately [13]. MS (mass spectrometry) identifies and quantifies mycotoxins based on their mass-to-charge proportion, and its performance (selectivity, sensitivity) can be upgraded if it is combined with techniques like liquid chromatography (LC); for example, in combination with LC, it is able to analyse several analytes at the same time, with a limit of detection of 5.8 mg/kg [12,13,17,38].

Liquid chromatography (LC) with UV detector is also helpful for patulin identification and quantification, but capillary micellar electrokinetic chromatography (MEKC) was found to be a quicker and more accurate method for its quantification [38].

Methods like liquid chromatography with tandem mass spectrometry (LC-MS/MS) or LC-MS [22] and gas chromatography-tandem mass spectrometry (GC-MS/MS) or GC-MS have also been greatly utilized in patulin analysis, but for these types of tools trained staff is indispensable [23]. LC allows the concomitant determination of multiple mycotoxins, without regard to their molecular structure or bioactivity. This technique can overcome the impediments of TLC, such as influence of temperature

and humidity. GC is harldly ever used in the mycotoxins' investigation as a consequence of the low volatility of analytes (derivatization is needed to increase their volatility) [17, 18].

GC and LC procedures have other limitations too, as they require expensive equipments and special competences [18].

In Belgium, a new LC-MS/MS method was developed, with a limit of quantification (LOQ) of 1.2 µg/l and limit of detection (LOD) of 0.4 µg/l for apple juice, along with 2.1 µg/kg and 0.6 µg/kg for apple puree. These values demonstrate the high sensitivity of the method [40].

Camara et al. (2023) developed a new method for patulin detection, a combination of µ-QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) procedure and HPLC-MS/MS. It was validated for linearity, selectivity, precision and accuracy. The sensitivity was evaluated based on LOD and LOQ, which were 0.32 and 1.15 µg/kg, lower than values presented by other authors. In addition, this technique displayed several other benefits, such as decreased sample loads, less washing steps and dangerous chemicals. It is a method with great potential [4].

Present focus is on immunological assays. Immunoassay methods', such as enzyme-linked immunosorbent assay (ELISA) and lateral flow immunoassay (LFIA) importance comes from their quick and reliable detection process, important for the food industry [17,23].

In contrast with the sensitive but complicated and expensive chromatographic techniques, ELISA is a rapid and simple, cost-effective, user-friendly screening method for mycotoxin analysis, based on antigen-antibody reactions, therefore, mycotoxins are recognized depending on antibodies' specificity and their capability to identify them [1]. It allows concomitant testing of numerous samples, it requires a small volume of sample and less tidy-up in comparison with chromatographic methods [38]. After spectrophotometric evaluation, the quantifiable result is obtained. The main disadvantage of this technique is that compounds with almost identical chemical structure can bound to the wrong antibodies and this can result in inaccuracy in the estimation (over- or underestimation) of toxin concentrations in examined samples [11,17].

Biosensors are also considered to be a functional tool for distinguishing mycotoxins in food products. They use biocomponents, such as enzymes, DNA or antibodies, to detect the toxins, which are biological analytes, in different food products [12,17]. Electrochemical-based biosensors have turned out to be great alternatives in detection of mycotoxins. The performance of these sensors has been improved by the increasing number of nanomaterials used in their design [11].

Another alternative to the traditional methods is real-time quantitative PCR (qPCR), a DNA-based technique. Because of its high sensitivity and specificity, the mould DNA target can be distinguished even in complex blends [23]. The disadvantage of it is that expert interpretation is required and that false negative or positive results can be distinguished only by primers [11].

A general aim is to lower the limit of detection and limit of quantification for patulin, target that was achieved by an innovative technique, invented by Erdem & Senturk (2024). They used a voltammetric aptasensor and a smartphone-integrated portable device: besides the success in lowering the limit of detection, high selectivity to patulin (selectivity studies were carried out with ochratoxin A, deoxynivalenol and fumonisin B1) was also accomplished [11].

Conclusions

It can therefore be concluded that patulin incidence in different products is a real problem that requires further attention. Since a great number of studies show that occurrence of patulin is higher in organic apple derivatives than in conventional ones, increased awareness is needed in the analysis of such derived products. The multitude of serious negative effects on consumer health highlights the need to apply the necessary investigation of the products that reach the market, knowing that apple juice or apple-based baby foods are consumed in almost every household. We have at our disposal a wide range of methods by which patulin detection can be performed, each with their own advantages and disadvantages, which can be applied depending on the situation, the equipment at hand and the competence of the personnel.

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