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Patulin Analysis: Sample Preparation Methodologies and Chromatographic Approaches

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1. Introduction

Patulin is a mycotoxin with carcinogenic, mutagenic and teratogenic potential mainly produced by several species of *Penicillium*, *Aspergillus* and *Byssoclamys*. This toxin has been found in fresh food such as fruits and vegetables, but may also be present in commercial apple juices and other food products. Food contamination with this toxin had led, over the years, to intense investigations related with its occurrence, toxicity, and also to develop prevention and detoxification methods from human and animal food chains. Patulin occurrence compelled several regulating entities to establish maximum limits in some food products, and thereupon, the identification and quantification of this mycotoxin demands the development of sensitive, selective and effective analytical methods.

2. Fungi and mycotoxins

Fungus is an organism which used to be traditionally included in the plant family, but nowadays belongs to the Fungi kingdom due to their considerable differences to plants, such as their inability to produce their own food. They are cosmopolitan in distribution, generally unnoticed due to small size. Nevertheless, these organisms have been applied in the food industry, used as sources of pharmaceutical agents for the treatment of infectious and metabolic diseases, and have shown high importance in agriculture due to their ability to establish symbiotic relationships with plants roots.

Fungi obtain their food through organic matter decomposition, creating at that stage an excellent opportunity for their growth in food. Fungal growth may result in production of toxins harmful to human health causing mycotoxicoses (Bennett, 1987).

In a brief chemical description, mycotoxins can be introduced as low-molecular-weight natural products, with great structural diversity, produced as secondary metabolites by fungi (Bennett & Klich, 2003). These fungi possess the ability to infect food, causing intoxication in animals and humans through contamination of crops or foods prepared from them, reason why the term mycotoxin comes from the fusion of the Greek word *mykes* and the Latin *toxicum*, meaning fungus and toxin, respectively. At this point, it is clear that mycotoxins are toxic compounds; but the definition of mycotoxin refers specifically to

metabolites toxic to vertebrates in low concentration (Bennett, 1987). Nevertheless, not all toxic compounds produced by fungi can be defined as mycotoxins, and a good example of this is the antibiotic penicillin, produced by *Penicillium* fungi, which is especially toxic to bacteria.

Common observation one can find in fungi kingdom is that mould species produces more than one type of mycotoxin, and the same mycotoxin can be produced by different types of moulds (Robbins et al., 2000, as cited in Ciegler & Bennett, 1980).

2.1 Toxicology and human health

Mycoses are infections caused by fungal growth on a host, and mycotoxicoses are intoxications, which occur as a consequence from different exposures sources such as food intake, respiratory and dermal contacts. Mycotoxicoses are similar to several other types of intoxications and their symptoms are quite dependent on the specific mycotoxin which causes the illness, the extent of exposure, age, gender and health of the individual, and other aspects like genetics and drug interactions. In the worst cases, mycotoxin poisoning can be amplified due to factors like sub nutrition, alcohol abuse and the presence of other diseases (Bennett & Klich, 2003).

Mycotoxicoses syndromes can be categorized as acute or chronic, and can range from rapid death to tumor development. Other less revealing diseases may occur when the mycotoxin interferes with immune system, leaving it susceptible to infectious diseases. The recognition of a mycotoxicoses is a long way to run. To do so, a dose-response relationship is required to recognize correctly the illness and associate it with the mycotoxin which caused it. Therefore, it is imperative to perform epidemiologic studies in the human population. The common way to execute these studies is by analogy, where a controlled animal population is subjected to a mycotoxin and human syndromes are reproduced. Human exposure to mycotoxins can also be determined by environmental or biological monitoring. In environmental monitoring, mycotoxins are measured in food, air, or other samples that may be in contact with the subjects, while in biological monitoring quantitative and qualitative evaluations of residues are made, and adducts and metabolites in tissue fragments, bodily fluids and excreta (Bennet & Klich, 2003, as cited in Hsieh, 1988) are evaluated.

Fungal diseases are considered to be a severe health problem worldwide.

2.2 Food contaminants

Nowadays, the use of botanical products is increasing due to the importance of diets and life style for human beings and, consequently, the quality of food products is fundamental. In developed countries, botanical products are recommended for medicinal and general health-promoting purposes. However, numerous occurrences of natural mycotoxins in botanicals and fruits have been reported, bringing along food spoilage and considerable economic losses.

Despite the development and industrialization of a country, mycotoxins can occur when environmental, social and economic conditions combined with meteorological conditions (humidity, temperature) promote growth of moulds. Therefore, from an ecological point of view, the role of mycotoxins in disease inducement should not be disregarded. For example,

the current trade patterns, mycotoxicoses resulting from contaminated food, locally grown or imported, could occur worldwide. And as consequence, it would be wrong to state that mycotoxin exposure only occurs in underdeveloped countries in which the population is malnourished and there is no regards in food handling and storage (Peraica et al., 1999).

The use of preventive techniques for the control of fungal growth in agricultural commodities and to improve methods for food preparation in industrial standards and food spoilage are never successful. For instance, taste, size, shape, texture and appearance are the basic features of evaluation of a fruit, these will dictate the fruit final destination, consumption or processing. In other hand, food products made from fruits are classified according to their quality using indicators of contamination. Specifically in food products, quality can be characterized by the growth of observable fungi, the existence of unpleasant odor and decomposition of the fruit. In the presence of a fungal contamination, a fruit can exhibit a profound change due to production of disintegrative pectinolytic enzymes responsible for the fruit tissues degradation.

As it was mentioned before, food contamination with mycotoxins is dependent of the influence of environmental and biological factors that determine the fungi production. These factors include the composition of the substrate, temperature, humidity, pH, microbial competition and insect damage (Songsermsakul & Razzazi-Fazeli, 2008; Sant'Ana et al., 2008). Contamination occurs during growing, harvesting, transportation, storage and/or processing of food products (Sant'Ana et al., 2008). As an example, *Aspergillus* and *Penicillium* species are usually associated with contamination during storage, whereas *Fusarium* species can produce mycotoxins before or right after harvesting (Abramson et al., 1997).

Contaminated feed for animals may also have deleterious effects on humans through food chain. In fact, the animal derived products like milk, eggs and meat may appear contaminated with chemicals produced by fungi (Shephard, 2008; Krska et al., 2008).

Beyond the devastating effect of agricultural growth of fungi, toxins produced by them have harmful effects on human health, including carcinogenicity, teratogenicity and growth retardation (Shephard, 2008). Aflatoxins, citrinin, fumonisin (Figure 1), ochratoxin, patulin, trichothecenes and zearalenone are among the mycotoxins that have received more attention in recent years due to their frequent occurrence and their effects on human health (Bennett & Klich, 2003).

2.3 Patulin

Patulin (Figure 2) was initially classified as broad-spectrum antifungal antibiotic. Later, it was found to inhibit more than seventy five different species of bacteria. Further investigations also revealed patulin's violent toxicity to animals and plants (Ciegler et al., 1971).

Patulin (PAT), IUPAC name 4-hydroxy-4H-furo[3,2-c]pyran-2(6H)-one, is characterized by being a hemiacetal lactone, with empirical formula $C_7H_6O_4$, molecular weight 154.12 g/mol, with an appearance of white powder and a melting point of 110°C. Patulin presents a very intense maximum UV absorption at 276 nm (Ciegler et al., 1971; Nielsen & Smedsgaard, 2003).

Fig. 1. Chemical structures of some common mycotoxins: aflatoxin B₁ (A), citrinin (B) and fumonisin B₁ (C).

Fig. 2. Patulin chemical structure.

Patulin is an organic compound very soluble in water and in most organic solvents. It is stable in diluted acids and resistant to temperatures at 125°C in the 3.5 - 5.5 pH range (Collin et al., 2008, as cited in Lovett & Peeler, 1973).

2.3.1 Sources and natural occurrence

The main species of fungi which produce patulin in food are *Aspergillus*, *Penicillium*, *Byssoschlamys*. *Penicillium expansum* has been identified as the major producer of patulin from the *Penicillium* species (Ciegler et al., 1971, as cited in Shibata et al., 1964) which is responsible for the vast majority of spoiled fruit. Both *Penicillium* and *Aspergillus* have the ability to grow at low temperatures. On the other hand, the fungi of the genus *Byssoschlamys* are responsible for patulin production in post-pasteurization stages. This production is due

to the fungi ability to survive the heat treatments which are applied to food products (Sant'Ana et al., 2008).

Fungal strains which produce patulin have been isolated over the years from various fruits and vegetables. Although patulin can occur in several fruits, grains and other foods infected by fungi, the main concern is with apples (Sommer et al., 1974, as cited in Brian et al., 1956; Harwig et al., 1973), apple cider (Sands et al., 1976, as cited in Stott & Bullernan, 1975) and apple juice (Scott et al., 1972) due to their higher consumption. It is also found and isolated from other fruits and vegetables; they are: grapes, pears, apricots (Sommer et al., 1974), cherries, strawberries, nectarines, raspberries, peaches, plums, tomatoes, bananas, almonds, hazelnuts and peanuts (Leggott & Shephard, 2001; Moake et al., 2005, as cited in Harvey et al., 1972; Buchanan et al., 1974; Lovett et al., 1974; Akerstrand et al., 1976; Andersson et al., 1977; Frank et al., 1977; Harwig et al., 1978; Brackett & Marth, 1979a; Jelinek et al., 1989; Jiminez et al., 1991; Prieta et al., 1994; Demirci et al., 2003; Ritieni, 2003).

Studies have been developed in several countries related with the occurrence of patulin; it has been identified in apples from Canada, England, United States, Australia (Sommer et al., 1974), South Africa (Leggott & Shephard, 2001), New Zealand (Moake et al., 2005, as cited in Walker, 1969) and Portugal (Gaspar & Lucena, 2009), and had also been found in apple juices from Canada (Moake et al., 2005, as cited in Scott et al., 1972), United States (Moake et al., 2005, as cited in Ware et al., 1974), Sweden (Moake et al., 2005, as cited in Josefsson & Andersson, 1976), South Africa (Leggott & Shephard, 2001), Turkey (Gokmen & Acar, 1998), Brazil (Moake et al., 2005, as cited in de Sylos & Rodriguez-Amaya, 1999), Austria (Moake et al., 2005, as cited in Steiner et al., 1999a), Italy (Moake et al., 2005, as cited in Ritieni, 2003), Belgium (Moake et al., 2005, as cited in Tangi et al., 2003), and Portugal (Gaspar & Lucena, 2009; Barreira et al., 2010).

2.3.2 Ecotoxicology and legal relevance

In the last decades, attempts have been made to carry out the work on the risk caused by patulin on human health. In the early 1940's, a work reported a metabolic compound derived from a *Penicillium* species, called patulin, which had the potential of being applied in the treatment to the common cold (Medical Research Council, 2004, as cited in Raistrick, 1943), but no evidence was found that patulin would be effective in the treatment of that disease. Due to its toxic evidenced effects, such as nausea, vomiting and gastrointestinal disturbances, patulin did not prove to be an efficient drug.

In the same decade, under the common name clavacin, a study of its antibiotic properties was performed. Preliminary results from this study showed that it was active against Gram-negative and Gram-positive bacteria, but it was also highly toxic to animal organisms (Katzman et al., 1944, as cited in Waksman et al., 1942).

Since then, several *in vitro* and *in vivo* studies have been performed to evaluate the toxicological risk associated with patulin consumption through fresh and processed food products, and other exposure types. In one of these chemical researches, patulin showed a strong affinity for sulfhydryl groups. The compound detains a great ability to form adducts with cysteine, although these adducts are less toxic than the unmodified toxin, respecting acute toxicity, teratogenicity, and mutagenicity studies. Its affinity for sulfhydryl groups explains the inhibition of many enzymes (Ciegler et al., 1976). Although the data on

genotoxicity were variable, most assays carried out with mammalian cells were positive, while assays with bacteria were mainly negative. After acute administration and short-term studies, the main signals of toxicity caused by patulin were gastrointestinal hyperemia, distension, hemorrhage and ulceration. *In vitro* and *in vivo* experiments had showed immunosuppressive properties at high dosages of patulin (Wouter & Speijers, 1996).

The International Agency for Research on Cancer (IARC) concluded that no evaluation was made respecting the carcinogenicity of patulin to humans and that there was inadequate evidence in experimental animals (IARC, 1986).

Even though no epidemic outbreak in humans and animals has been attributed to patulin contamination, increasing concern with public health forced the regulating authorities to establish maximum limits. As a consequence, international recommendations and regulations were established for patulin maximum levels allowed in food products; patulin content warnings in food products have to be displayed, acting like quality markers. Within European countries the regulation (EC) No 1425/2003 was adopted and setting as maximum levels of 50 µg/L for fruit juices and derived products, 25 µg/L for solid apple products and 10 µg/L for juices and foods aimed for babies and young infants (Commission regulation (EC) No 1881/2006). Joint FAO/WHO Expert Committee on Food Additives (JECFA) also established the provisional maximum tolerable daily intake 0.4 µg/kg body mass/day (JECFA, 1995). Nowadays, the US Food and Drug Administration (FDA) limit patulin to 50 µg/L.

2.4 Analysis

Patulin analysis encompass several important steps: sampling, sample preparation, isolation and/or identification, quantitation and, sometimes, statistical evaluation. Each step is equally important to obtain good results to fulfill the analytical purpose.

Sampling includes deciding on sampling points and choosing a method to get appropriate amounts of samples, which can be solid or liquid with very different structural and chemical complexity.

2.4.1 Sample preparation techniques

Most of the time, sample preparation is necessary to isolate and/or concentrate patulin from sample matrices, having into account its chemical properties and the complexity of matrices. As mentioned before, patulin can be found in human food and beverages, animal feed, biological and environmental samples.

Matrix solid-phase dispersion (MSPD) was reported (Wu et al., 2008, 2009) as a suitable method to extract patulin from apples. A small portion of apple was blended homogeneously in a C₁₈-bonded silica solid support. After the washing step with hexane, patulin was extracted by elution with dichloromethane. After solvent evaporation, the extract was dissolved in acetic acid buffer solution and the patulin was analyzed by High Performance Liquid Chromatography (HPLC).

The Association of Official Analytical Chemists (AOAC) adopted a liquid-liquid extraction as sample preparation methodology for patulin analysis of apple juices and concentrates in its Official Method 995.10 (Sewram et al., 2000, as cited in Brause et al., 1996). The method

includes an initial multi-extraction step with ethyl acetate and a posterior extraction with an aqueous sodium carbonate solution. The combined extracts were dried and evaporated, then being dissolved in acetic acid solution and chromatographed for patulin analysis. Several improved methodologies of the official method have been reported over the years (Gökmen & Acar, 1998; Iha & Sabino, 2006). One of them was described for cloudy apple juice and apple purees, and includes a pretreatment procedure with pectinase to improve the clarity of juices and purees before patulin analysis (MacDonald et al., 2000).

2.4.1.1 Solid Phase Extraction (SPE)

Solid phase extraction (SPE) emerged as an extremely attractive versatile technique for sample preparation of patulin containing matrices. The principles of the technique are based on chromatography: solutes are dissolved or suspended in a liquid and eluted through a solid phase; depending on their affinity, a separation occurs (Figure 3). SPE has been used to clean-up matrices and concentrate trace substances, such as patulin. Like any other technique it has also limitations: matrix effect and the undesired competition between analyte(s) and the other matrix components. As can be seen in Figure 3, SPE uses pre-packed cartridges containing up to 500 mg of stationary phase - silica or silica bonded to specific functional groups such as hydrocarbons of variable chain length, reversed phase SPE, or quaternary ammonium or amino groups, anion-exchange SPE, or sulfonic acid or carboxylic groups, cation-exchange SPE.

Fig. 3. SPE procedure steps (Supelco, 1998).

For patulin analysis in apple juices (Gökmen et al., 2005) two SPE systems using a tandem polyvinylpyrrolidone-octadecyl (PVPP-C18) cartridge and a hydrophilic lipophilic balanced (HLB) macroporous copolymer sorbent cartridge, were described. The technique required few mL of juice and the elution was done with diethyl ether. After solvent

evaporation the residue was dissolved in a mixture of acetonitrile/water with a small portion of acetic acid and then chromatographed.

Another work reported the patulin analysis of infant's apple-based products involving a SPE cleanup step using a unconditioned silica gel cartridge (Arranz et al., 2005).

For the analysis of an apple juice syrup it was described a pretreatment procedure using C₁₈-SPE cartridges (Li et al., 2007). The syrup sample was diluted with acetic acid buffer solution and loaded onto the column. After the washing procedure using hexane, the cartridge was dried with a strong stream of air for 15 min, and eluted with the solvent mixture (hexane/ethyl acetate/acetone) in a gradient mode. After acidification, patulin was analyzed by HPLC.

2.4.1.2 Microextraction by packed sorbent or packaged syringe (MEPS)

The development of miniaturized analytical techniques has been done to fulfill many requirements and also enable rapid analysis at lower operation costs, with lower environmental pollution problems. Microextraction by packed sorbent or packaged syringe (MEPS) methodology seems to be a very promising solution in patulin analysis. MEPS technique uses a small amount of stationary phase, packed in the drum called BIN (Barrel Insert and Needle Assembly, Figure 4) of a gastight syringe (100µL – 250µL). Like SPE, MEPS aims the elimination of interferences and the selective isolation and concentration of the target compounds, but in a micro scale. MEPS procedure steps are similar to SPE laboratorial approaches and are illustrated in Figure 5.

Fig. 4. MEPS Syringe and BIN (SGE, 2009).

Fig. 5. MEPS procedure steps (SGE, 2009).

Using MEPS as sample preparation technique, a new methodology for patulin analysis is described, for the first time, in this book chapter (Section 2.4.3.2). It aims the identification and quantification of this mycotoxin in real matrices.

2.4.2 Chromatographic methodologies

The most suitable analytical methods respecting accuracy, low detection limits and simple procedures for patulin analysis in food products, especially apple juice and its derivatives, involve chromatographic techniques such as thin-layer chromatography (TLC), gas chromatography (GC) and high-performance liquid chromatography (HPLC).

The pioneering methodology used in the identification and quantification of patulin, in apple juice, was thin-layer chromatography (TLC) with the advantages of being simple and low cost. One official method of the Association of Official Analytical Chemists (AOAC) for the analysis of mycotoxins (1974) uses TLC followed by silica gel column chromatography as sample preparation procedures. The analysis was carried out in silica gel plates, using, for detection, the reaction of patulin with 3-methyl-2-hydrazone benzothiazolinone and HCl (MBTH). The limit of detection (LOD) was described as 20 µg/L (Shephard & Leggott, 2000, as cited in Scott, 1974). Alternative detection and quantification methods were described using fluorodensitometry (Shephard & Leggott, 2000, as cited in Duraković et al., 1993), or absorbance-transmittance followed by densitometry at 275nm (Shephard & Leggott, 2000, as cited in Leming et al., 1993). Reversed phase TLC was also investigated, but despite the patulin elution with a wide variety of solvents, the technique was not applied to contaminated samples (Shephard & Leggott, 2000).

HPLC coupled with ultraviolet (UV) or photodiodes (DAD) detection is the most used chromatographic technique for patulin analysis; it allows an easy identification and quantification of patulin through its characteristic absorption spectrum. The AOAC also adopted HPLC-UV method (official method 995.10), for the detection and quantification of patulin in apple juices. As mentioned before, liquid-liquid extraction was used as sample preparation technique. The HPLC was done using a C₁₈ reversed phase column, with particle size of 5 µm and pore size ranging from 12 to 25 nm, and isocratic elution with a flow of 1mL/min, and the eluent was composed by acetonitrile in acidified water. UV detection at 276 nm (Moake et al., 2005, as cited in AOAC official method 995.10) was used. The LOD achieved is low (5 µg/L).

Improved AOAC methods were described in the literature for the determination of patulin in apple juices (Gökmen & Acar, 2005) and in apple solid derivatives (Katerere et al., 2008). Over the years, HPLC methodologies associated with mass spectrometry (MS) were also developed. A work published in 2000 described patulin detection by collision-induced dissociation (CID) using atmospheric pressure chemical ionization (APCI); the LOD was good, with values near 4 µg/L (Sewram et al., 2000).

Gas chromatography (GC) has also been applied in the analysis of patulin. The literature reported GC-MS analysis by electron impact ionization, using a silylated patulin derivative (Moukas et al., 2008). Raw patulin (not derivatized) was also determined in apple juice by GC-MS using negative chemical ionization (Roach et al., 2000). Other GC methods were reported using on-column injection and selected ion monitoring (SIM) detection (Moake et al., 2005, as cited in Llovera et al., 1999).

A protocol including biphasic dialysis extraction, *in situ* acylation, as sample preparation and GC-MS (SIM) analysis was developed for detection and quantification of patulin (acetylated) in fruit juices (Sheu & Shyu, 1999). Another work presented the detection and quantification of C¹³ labeled patulin (Rychlik & Schieberle, 1999).

Capillary electrophoresis (CE) was also described as an alternative method for patulin analysis. In 2000, a capillary electrophoresis method was published as an analytical tool for rapid and highly sensitive analysis of patulin in cider; a fused silica capillary column was used, and the elution was done with an aqueous. The separation was achieved by migration of charged particles in the buffer. The cations migrated to the cathode and anions migrated to the anode under the influence of an electro-osmotic flow (EOF). In fact, compounds or mixtures of neutral and charged compounds can be analyzed by micellar electrokinetic capillary electrophoresis or micellar electrokinetic capillary chromatography (MECC). The method was described as having the advantage of requiring small amounts of sample and smaller amount of organic solvents when compared with HPLC (Tsao & Zhou, 2000).

2.5 Development of improved analytical methodologies

For patulin analysis, two sample preparation procedures using SPE and MEPS were developed and associated with an improved reversed-phase HPLC methodology (Gaspar & Lucena, 2009). The purpose was to simplify the sample, liberating the patulin from its original matrix, and allowing therefore a better performance of the overall analytical method. Table 1 shows the analytical parameters of the HPLC methodology.

Retention time (min)	Linearity			LOD (µg/L)	LOQ (µg/L)	Precision (RSD,%), n = 4
	Range (mg/L)	Calibration equation	R ²			
16.8	0.03 – 0.50	y = 136592x-2161.5	0.9996	2.0	6.0	3.5

Table 1. Analytical parameters of improved HPLC methodology for patulin analysis (Elvira & Lucena, 2009).

2.5.1 SPE-HPLC/DAD methodology

Aiming a small scale sample preparation procedure, a previously described SPE approach (Li et al., 2007) was improved and optimized. The purpose was the analysis of patulin in fresh fruits, namely apples, but also in apple juices commercialized in Portugal. The SPE sample preparation optimization was done by spiking fresh (healthy) apples extracts with patulin, in order to establish the best SPE analytical conditions, such as the choice of stationary phase and its conditioning, washing and extraction steps.

The SPE optimized sequential procedure is:

1. Using C₁₈-SPE cartridges, pre-washed with (3 mL) methanol, methanol:water (3 mL, 10% methanol) and acidified water (3 mL MilliQ water-perchloric acid ((100):0.01,(v/v))) sequentially (illustrated in Figure 3, steps 1 and 2);
2. The column was not allowed to run dry;

3. The sample (0.5 mL) was introduced and eluted at a flow rate of 2–3 mL/min (Figure 3, step 3);
4. The following washing step was done with (0.5 mL) acidified water (Figure 3, step 4);
5. Sample elution was done with (3 × 1 mL) methanol (Figure 3, step 5);
6. The combined eluates were evaporated to dryness under vacuum;
7. After dissolution in (0.5 mL) acidified water the sample was analyzed using the previously mentioned improved HPLC methodology (Gaspar & Lucena, 2009);

This improved sample preparation changed the previously described (Li et al., 2007) washing solvent - acidified water instead of hexane - and also the eluent composition, methanol, a greener solvent, instead of the mixture hexane/ethyl acetate/acetone.

This optimized SPE-HPLC/DAD methodology showed an average recovery of 82% with a RSD value of 6% in the linear dynamic range 200 to 600 ppb (Table 2). The precision of the method showed a repeatability of 1.2% and a reproducibility value of 2.2%. According to the literature (Miller & Miller, 1988; IPQ, 2000) being these values below 10%, they indicate a good method performance. The overall SPE-HPLC methodology represents an economical, faster and routine usable methodology.

Range (µg/L)	Recovery (RSD,%), n = 3	Repeatability (RSD,%), n = 3	Reproducibility (RSD,%), n = 3
200 - 600	82 (6)	1.2	2.2

Table 2. Analytical parameters of improved SPE-HPLC methodology for patulin analysis.

2.5.2 MEPS-HPLC/DAD methodology

Using the recent MEPS analytical tool (Abdel-Rehim, 2004), a new, simple, sample preparation method was developed for patulin analysis. MEPS methodology can be rationalized as a miniaturization of SPE analytical system; it employs smaller quantities of sample and eluent (few microliters), being also adequate to remove interferences, simplifying the analysis.

For patulin analysis, this new sample preparation methodology was performed using the following sequential steps:

1. MEPS was carried out by means of a SGE Analytical Science (SGE Analytical Science, Germany) apparatus, consisting of a 250 µL HPLC syringe with a removable needle; the syringe was fitted with a BIN (Barrel Insert and Needle) containing the C₁₈ sorbent and was used to draw and discharge samples and solutions through the BIN (Figure 4);
2. The sorbent was activated/conditioned three times with (3 × 100 µL) methanol and (5 × 100 µL) MilliQ water-perchloric acid ((100): 0.01 (v/v)) (Figure 5);
3. Sampling was done using a volume of 2x25 µL of sample solution, being introduced through the stationary phase for three times, in order to remove the interferences and retain the patulin (illustrated in Figure 5, step 1);
4. The analyte was eluted using methanol (2 × (3 × 25 µL)) and after analyzed by HPLC using the previously described conditions (Gaspar & Lucena, 2009) (illustrated in Figure 5, steps 3 and 4);

This method development evidenced the relevance of several factors in this sample preparation methodology, MEPS: sampling speed was around 10 µL/s, sample introduction

was done in a fractionated mode ($2 \times 25 \mu\text{L}$) and the washing step was not used; these procedures revealed a better recovery. The extracting solvent was of crucial importance - methanol was much better than acetonitrile for the patulin analysis.

The method performance was achieved using five replicates, spiking the target samples (apples) with different quantities of patulin standard ranging from 200 to 600 ppb (Table 3). The average recovery was 69% with a RSD of 4%. The precision of the method showed a repeatability of 3.2% and a reproducibility value of 4.0%. Also here these values are below 10%, indicating a good precision for this new sample preparation methodology (Miller & Miller, 1988; IPQ, 2000). MEPS can be an excellent alternative to the SPE technique, being faster, less solvent consuming and less expensive than SPE.

Range ($\mu\text{g/L}$)	Recovery (RSD,%), n = 5	Repeatability (RSD,%), n = 5	Reproducibility (RSD,%), n = 3
200 - 600	69 (4)	3.2	4.0

Table 3. Analytical parameters of MEPS-HPLC methodology for patulin analysis.

2.5.3 Analysis of food matrices

Two food matrices: an infected apple and a commercial apple juice were analyzed using both sample preparation methodologies: SPE-HPLC/DAD and MEPS-HPLC/DAD. These analyses are shown in Figures 6 and 7.

Fig. 6. SPE-HPLC/DAD (A) and MEPS-HPLC/DAD (B) patulin analysis from a naturally infected apple; the UV spectrum is shown (conditions described in Section 2.4.3.1 and 2.4.3.2).

As it was mentioned before, patulin is formed in bruised apples as a result of contamination with fungi. The analysis of an infected (mouldy) apple revealed that it contains a very high amount of patulin (894 ppm in SPE-HPLC/DAD and 919 ppm in MEPS-HPLC/DAD), much higher than its LD₅₀ (5 mg/Kg = 5 ppm). This result indicates how dangerous is the direct consumption of damaged fruits and their use in the production of fruit juices (Figure 6).

Fig. 7. SPE-HPLC/DAD (A) and MEPS-HPLC/DAD (B) patulin analysis from a spiked commercial apple juice; spike 7.7 ng /50 μ L (conditions described in Section 2.4.3.1 and 2.4.3.2).

Apple juice (cloudy) was also analysed by both methods showing a level of patulin lower than 6 ppb, the limit of quantification (LOQ) of both methods for this compound. Spiked matrices (Figure 7) were also analyzed, by both methods, to test the methodologies. The results revealed that the analyzed juice was produced with good sanitation criteria, satisfying the legislation limits (Commission Regulation (EC) No 1881/2006). These results show the necessity of constant surveillance of the occurrence of this toxin in fruit juices and evidenced that national public institutions should be capable of evaluating and determining food content in toxic substances in real time, due to the risk associated with consumption.

3. Conclusions

Patulin analysis is an important subject with social relevance. This chapter describes the most suitable analytical methods respecting accuracy, low detection limits and simple procedures for application in quality control, developed for the determination of patulin in food products, especially apple juice and its derivatives. Techniques such as thin-layer chromatography (TLC), gas chromatography (GC) and high-performance liquid chromatography (HPLC) were described together with sample preparation methodologies like liquid-liquid extraction (LLE) and solid-phase extraction (SPE) to determine the patulin. This chapter also describes, for the first time, an improved, simple, no time consuming, trace analysis micro extraction in packed syringe (MEPS) methodology for the determination of patulin in food products, and compares and discusses the use of MEPS and SPE in patulin extraction.

Having into account the importance of detecting this mycotoxin in food chain as marker of quality, this work will contribute to a better characterization and quantification of its presence in human diet and will make possible to determine the toxicological relevance of human exposure to patulin, its biological role and long-term implications of its consumption for human health.

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This is a good book on upcoming areas of Ecotoxicology. The first chapter describes genotoxicity of heavy metals in plants. The second chapter offer views on chromatographic methodologies for the estimation of mycotoxin. Chapter three is on effects of xenobiotics on benthic assemblages in different habitats of Australia. Laboratory findings of genotoxins on small mammals are presented in chapter four. The fifth chapter describes bioindicators of soil quality and assessment of pesticides used in chemical seed treatments. European regulation REACH in marine ecotoxicology is described in chapter six. X-ray spectroscopic analysis for trace metal in invertebrates is presented in chapter seven. The last chapter is on alternative animal model for toxicity testing. In conclusion, this book is an excellent and well organized collection of up dated information on Ecotoxicology. The data presented in it might be a good starting point to develop research in the field of ECOTOXICOLOGY.

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