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Review

Chromatographic determination of the mycotoxin patulin in fruit and fruit juices

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Abstract

Patulin is a mycotoxin produced by several fungal species of the genera *Penicillium* and *Aspergillus*, but principally by *Penicillium expansum* on fruit such as apples. The occurrence of patulin as a natural contaminant of apple juice is a worldwide problem and international recommendations and regulations have been made for maximum levels permitted in consumer products. This paper reviews currently available analytical methods for its determination in fruit and fruit juices. Of these, HPLC with ultraviolet or, preferably, photodiode array detection is most widely used, although GC and TLC methods have also been described.

Keywords: Reviews; *Penicillium spp.*; Fruits; Fruit juices; Food analysis; Patulin; Mycotoxins

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1. Introduction

Patulin {4-hydroxy-4*H*-furo[3,2-*c*]pyran-2(6*H*)-one} is a toxic secondary metabolite produced by a wide range of fungal species of the *Penicillium* and *Aspergillus* genera, of which *P. expansum*, a common contaminant of damaged fruit such as apples, is the most important [1,2]. Patulin has been found as a natural contaminant of processed apple products and it has been suggested that its presence may be indicative of the quality of the fruit used in production [3].

Patulin is a toxic lactone possessing antibiotic properties and has an LD_{50} (ip) in mice of 5 mg/kg [4]. The need for validated analytical methods has been highlighted by its natural occurrence in fruit

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juices (particularly apple juice) and recent reviews of its toxicology. Although patulin failed to give any indication of mutagenic potential in *Salmonella typhimurium* bacteria in the Ames test, it has been shown to produce chromosomal damage [5,6]. The UK Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment has classified patulin as mutagenic [7]. A recent review of the patulin toxicological data by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) concluded, based on available evidence, that patulin has no reproductive or teratogenic effects, but does show embryotoxicity accompanied by maternal toxicity [8]. At relatively high doses, patulin has immunosuppressive properties [8], although a recent study in mice based on realistic human exposures failed to demonstrate any immunotoxicity [9]. Based on available experimental results, it was concluded that patulin is genotoxic, but that no adequate evidence existed for carcinogenicity in experimental animals [8]. In order to establish guidelines for human exposure to patulin, JECFA recently lowered the provisional maximum tolerable daily intake (PMTDI) for patulin from 1 to 0.4 μg/kg body mass/day based on a no observed effect level (NOEL) of 43 μg/kg body mass/day and the use of a 100-fold safety factor. National and international groups have recommended that apple products intended for human consumption should not contain residual patulin levels above 50 μg/kg (ppb) [10], while a number of countries regulate patulin in juice at levels ranging between 20 and 50 μg/l [11].

Although mention will be made of earlier methods, this review of patulin analytical methods will concentrate on the advances that have been made in the past 10 years. In view of the increasing concern that arises from the presence of toxins in laboratory wastes, attention is drawn to the use of either ammoniation or alkaline potassium permanganate oxidation for the safe removal of patulin from such wastes or for the detoxification of spills [12].

2. Thin-layer chromatographic methods

The early methods for the determination of patulin in apple juice utilised TLC and the original Association of Official Analytical Chemists (AOAC) official method was approved based on a collaborative study by Scott in 1974 [13]. This TLC method (currently method 974.18) involved ethyl acetate extraction and clean up on a silica gel column. Detection on silica gel plates was achieved by spraying with 3-methyl-2-benzothiazolinone hydrazone (MBTH) with a detection limit of approximately 20 μg/l. A review of patulin methodology in 1986 concluded that the TLC methods had already given way to a predominance of HPLC techniques as these were less time consuming, gave improved resolution from the common contaminant, 5-hydroxymethylfurfural (HMF) and achieved improved sensitivity [14]. Despite this shift to more sophisticated methodology, a few workers have further developed TLC techniques with alternative clean up methods, TLC plates and detection methods. The use of diphasic dialysis for extraction of patulin from juice was demonstrated [15]. In this technique, a membrane containing the organic extraction solvent is stirred or shaken in the aqueous matrix such that the low molecular weight analytes of interest pass through the membrane, while the higher molecular weight impurities are retained in the aqueous matrix. Patulin in apple juice was extracted by this technique, and determined without extract clean up by TLC on silica gel plates with MBTH as spray reagent and with quantification by densitometry [16]. The method achieved extraction recoveries of 65% and had a detection limit of 50 μg/l.

The potential use of reversed-phase TLC plates was investigated by Abramson et al. for a wide range of mycotoxin standards, including patulin, with different mobile phase solvent mixtures [17]. Although patulin could be successfully chromatographed on these plates, the technique was not applied to naturally contaminated samples. Alternative methods for quantification of the patulin spot on the TLC plate have also been reported. Lin et al. used densitometry in absorbance–reflectance mode at a wavelength of 275 nm to quantify the spot intensities of patulin extracted from a maize matrix [18], while Durakovic et al. used fluorodensitometry following the formation of fluorescent derivatives obtained by exposure of the patulin chromatographic spot to concentrated ammonia fumes [19]. This method formed the basis of an analytical method for

patulin in apple juice which had a detection limit of 100 μg/l.

3. Gas chromatographic methods

Although HPLC methods for the determination of patulin have mostly been preferred, a number of GC methods have been developed. These have generally involved the formation of trimethylsilyl ether derivatives with detection by electron-capture or mass spectrometry. Recent work has described the preparation of heptafluorobutyrate (HFB) derivatives from the corresponding imidazole [20]. Chromatographic separation was achieved on a non-polar fused-silica capillary column with electron capture detection. The response was linear over the range 0.05–0.5 ng injected on column and was also highly reproducible (RSD of 1.9–4.0%). Application of this technique to naturally contaminated apple juice, which was extracted with ethyl acetate and cleaned up by silica gel column chromatography, gave a detection limit of 10 μg/l.

A direct acylation procedure prior to sample extraction has been described for the analysis of patulin in which the resulting patulin acetate was determined by GC with MS detection in the selected ion monitoring mode with a detection limit of 10 μg/l [21]. These same authors have also developed an in situ acylation method in which the patulin is extracted by diphasic dialysis into dialysis tubing containing methane chloride as solvent, acetic anhydride as derivatising agent, 4-*N*,*N*-dimethylaminopyridine as derivatization activator and nitrobenzene as internal standard [22]. The dialysis tubing was stirred in the juice sample for 24 h at 25°C. The organic phase in the tubing was then removed and the patulin acetate was chromatographically separated by GC using MS detection with selected ion monitoring of fragments formed by electron impact ionization. Although the time period for extraction is lengthy, the remainder of the method is rapid and can achieve an estimated limit of quantification of 10 μg/l with a mean recovery of 79%.

Although previous methods have utilised patulin derivatives to ensure adequate chromatographic separation and detection, a recent publication has described the use of electronic pressure control with on-column injection for analysis of underivatised patulin [23]. Based on MS detection, this method achieved a detection limit of 4 μg/l in apple juice.

4. High-performance liquid chromatographic methods

Although the TLC and GC methods for patulin determination are in routine use in some laboratories, the majority of laboratories that participated in a proficiency testing exercise reported the use of HPLC methods [24]. HPLC, coupled with UV detection, is particularly well suited to the determination of patulin, since the toxin is relatively polar and exhibits a strong absorption spectrum. Although some of the original HPLC work involved normal-phase chromatography [25], all recent publications describe the use of reversed-phase columns. Ethyl acetate has been the universal extraction solvent, whereas various clean up systems have been developed over the years.

A method for patulin in apple juice published by Tanner and Zanier in 1976 involved ethyl acetate extraction and subsequent purification by solvent partition with a solution of sodium carbonate for the removal of some potentially interfering phenolic compounds [26]. This method has undergone slight modifications, but forms the basis of currently recommended HPLC methods. It was slightly modified by Möller and Josefsson to enable the clean up to be performed on a microscale [27], whereas Forbito and Babsky published a modification to the HPLC mobile phase for improved resolution of the patulin [28]. In its original form, the method formed part of a collaborative study in which the performance of two reversed-phase HPLC methods for the determination of patulin in apple juice were evaluated in twelve laboratories from ten countries [29]. The second method, published by Stray [2], differed from the former in that the purification of patulin from an ethyl acetate extract was achieved with silica gel column chromatography. The detection limits of the methods were considered to be 5 μg/l and mean recoveries were 78.4% for the method of Stray [2] and 81.4% for the method of Tanner and Zanier [26]. Respective RSDs were 7.3% and 15%. Subsequently, the methods of Forbito and Babsky [28] and of

Möller and Josefsson [27] were slightly modified with respect to solvent usage in the laboratories of the US Food and Drug Administration and Analytical Chemical Services of Colombia, Inc. [30]. The resulting optimised method was the subject of an international collaborative study performed under the auspices of AOAC International in which 22 participants analysed test samples spiked with 20, 50, 100 and 200 μg patulin/l, as well as a naturally contaminated test sample containing about 31 μg/l [30]. Recoveries of patulin ranged from 91 to 108% with a mean of 96%. Within-laboratory repeatability values ranged from 10.9 to 53.8%, while between-laboratory reproducibilities ranged from 15.1% to 68.8%. Based on these results, the method was adopted first action by AOAC International (method 995.10) [30].

Solid-phase extraction (SPE) technology has recently been used for the extraction and/or purification of patulin from apple juice. Rovira et al. developed a method using a silica SPE cartridge for purification of the ethyl acetate extract prior to reversed-phase HPLC [31]. The diphasic dialysis membrane extraction technique that was previously applied to sample clean up for semiquantitative TLC analysis [15,16], was further developed to include a silica SPE cartridge clean up of the extract coupled with reversed-phase HPLC determination with UV detection [32]. This method was applied in a survey of patulin levels in commercial apple juice and apple food samples purchased in Madrid, Spain [33]. In another sample preparation technique, a commercial multifunctional cleaning column (MycoSep column, Romer Labs., Union, MO, USA) was used to extract patulin from a mixture of juice (or juice concentrate) and acetonitrile [34]. The column retained interfering impurities, while the patulin passed through the column so as to give an analytical recovery of 82–96%. Another technique used a more conventional reversed-phase SPE cartridge to retain patulin. The undiluted apple juice was applied directly to a preconditioned cartridge containing a copolymer of divinylbenzene and *N*-vinylpyrrolidone (Oasis cartridge, Waters, Milford, MA, USA) [35]. After washing the column, patulin was eluted and determined by HPLC with an analytical recovery of 93–104%. Yet another method for patulin extraction and purification was developed for analysis of French ciders in order to eliminate interfering tannins and phenolic compounds [36]. Patulin was determined after extraction on an Extrelut (Merck, Darmstadt, Germany) extraction column with subsequent purification on a Sep-Pak (Waters) Florisil SPE cartridge followed by reversed-phase HPLC with UV detection. Analytical recoveries ranged from 90.4% to 91.7% and the limit of quantitation was 10 μg/l.

Although the potential for interference in the HPLC determination of patulin by HMF, which generally elutes from a reversed-phase column just prior to patulin, has been recognised for some time [14,37], a recent publication has reported the simultaneous quantification of both substances using the same extract with an overall recovery for HMF of 94% and for patulin of 103% [38]. The limit of detection for HMF was reported as 10 μg/l as opposed to that of patulin of 5 μg/l. Of particular importance in the HPLC analysis of processed apple juices is the separation of patulin from intrinsic phenolic compounds in general and HMF in particular. The use of photodiode array detection to distinguish patulin spectrally from these co-extracted compounds has considerable application in providing confirmation of the purity of the chromatographic peak [39].

Patulin is a low-molecular-mass, polar molecule which is only retained on reversed-phase HPLC columns by the use of mobile phases with high aqueous content. The mobile phases used by most analysts are mixtures of water and acetonitrile (up to 10%) or water and tetrahydrofuran (up to 5%). The exact composition is usually adjusted to achieve adequate separation and retention time [30]. The HPLC is generally operated in the isocratic mode, although problems can be experienced in certain apple products with late eluting impurities. To overcome this problem, some analysts have used gradient conditions [35,40].

5. Future developments in patulin analysis

As in other areas of mycotoxin analysis, both rapid clean up and analytical methods based on immunochemical technology are under development. In this regard, the production of suitable antibodies for use in an enzyme-linked immunosorbent assay

(ELISA) or for coupling on an immunoaffinity (IA) clean up column is required. To date, no commercial products have been marketed. The production of polyclonal antibodies against patulin hemiglutarate has been described and these have been tested in an indirect ELISA [41]. However, these authors failed to clearly demonstrate the production of specific antibodies against patulin alone and suggested that monoclonal antibody production and purification might produce a more sensitive and specific ELISA.

Recent work has described the synthesis of a ^{13}C-labelled patulin ([$^{13}C_2$]-patulin) to be used in a stable isotope dilution assay [42]. The production and use of this labelled product as an internal standard for mass spectrometric detection allows for the determination of patulin in samples requiring more extensive clean up procedures than currently employed. Based on high resolution mass spectrometric detection following GC separation of the trimethylsilyl derivative of patulin, a detection limit as low as 12 ng/l was achieved using this stable isotope as an internal standard [43].

6. Conclusion

The HPLC method with UV detection is fast and reliable, and has been applied to a number of different matrices. As such, it is the general method of choice for routine determination and monitoring of patulin levels, although TLC and GC methods are available. The use of photodiode array detection in place of single wavelength detection adds confidence to the analytical result due to the ability of this detector to provide spectral confirmation for patulin. Future developments will probably be aimed at achieving reliable confirmation of low levels of patulin by mass spectrometry and at the development of specific patulin antibodies for use either in enzyme-linked immunosorbent assays or in immunoaffinity clean up columns.

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