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Determination of patulin in apple juice by high-performance liquid chromatography with diode-array detection

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Abstract

A method is described for the detection of patulin in apple juice and the simultaneous determination of the phenolic composition. Spectral data obtained with diode-array detection showed that patulin can be easily distinguished from compounds eluting under the same conditions. The detection limit for patulin was 8.96 μg/l.

1. Introduction

Patulin (Fig. 1) is a mycotoxin produced by several species of moulds (*Penicillium*, *Aspergillus* and *Bissochlamys*) [1]. It is particularly produced by the apple-rotting fungus *Penicillium expansum*, thus being of toxicological concern in apple products such as apple juice. However, patulin has also been detected in grape juice [2], barley, wheat and corn [3] and with growth of fungus in pears, bananas, pineapples, grapes, peaches and apricots [4].

There is increasing interest in developing methods for the detection and determination of patulin in foodstuffs, owing to its toxic activity and its teratogenicity, carcinogenicity and mutagenicity [5] as tested in laboratory animals. No data are available about the toxicity of patulin in humans, but some governments (Switzerland, Sweden, Belgium, Russian Federation and Norway) have established a maximum permitted concentration of 50 μg/l in apple juice [5,6].

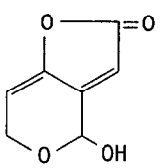

Fig. 1. Structure of patulin, 4-hydroxy-4*H*-furo[3,2-*c*]pyran-2(6*H*)one.

Although patulin is removed from juices during fermentation [2,7], its level is only decreased by about 20% during common processes in fruit-juice production [8]. Patulin is therefore a good indicator to detect the quality of the fruit used in the manufacture of juices. For these reasons, a number of methods have been developed for detecting and determining patulin in fruit juices. TLC methods have been rapidly replaced by HPLC [2,5,6,8–14].

Sample preparation involves two steps; (a) extraction with ethyl acetate and (b) a purification step, such as partitioning with aqueous sodium carbonate solution [10,13], elution through a silica gel [5,8,11,12] or Extrelut [2,9]

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column or the use of a Sep-Pak cartridge [6]. In HPLC, reversed-phase columns are employed with different solvents, with UV detection between 272 and 280 nm.

All of the reported methods have the unique purpose of detecting patulin and none of the studies consider the compounds eluting under the same conditions. Only Leuenberger *et al.* [9] and Lehmann and Wald [11] reported that 5-hydroxymethyl-2-furaldehyde (a compound formed in thermal treatments) elutes under the same conditions as patulin.

In this paper we present a method for the detection and determination of patulin and also phenolic compounds naturally occurring in fruits. These have proved to be useful for characterizing fruit juices [14] and for detecting adulteration [15,16]. The method involves HPLC with diode-array detection (DAD). Patulin can be distinguished from phenolics in contaminated apple juice by its chromatographic and spectral features. The study of different parameters obtained from spectral data [17] permits the differentiation of patulin from over 60 low-molecular-mass phenolic compounds.

2. Experimental

2.1. Sample preparation

Different amounts of a solution of patulin [2.25 mg/l in acetonitrile–water (80:20, v/v)] were added to commercial apple juices that contained no traces of patulin. After being homogenized, the samples were extracted. Previous tests showed that the extraction of patulin with ethyl acetate is more efficient than that with diethyl ether, so the initial method for extracting non-flavonoid phenolics and 3-flavanols from fruit juices [14] was modified. The juices were not concentrated, but only ethyl acetate was used, and the number of extractions, the ratio of organic phase to the aqueous phase and the final volume of the sample were changed, in order to increase the recovery of patulin. No qualitative differences were found between the phenolic profiles of an apple juice extracted by the two methods. Therefore, samples (25 ml) were extracted six times with ethyl acetate (20 ml). The organic solutions were combined, dried for 30 min with anhydrous sodium sulphate, filtered and evaporated to dryness. The residue was dissolved in 1 ml of methanol–water (50:50, v/v) and 10 μl were injected into the HPLC apparatus. Samples were prepared and analysed in triplicate.

2.2. HPLC conditions

The equipment was obtained from Waters (Milford, MA, USA): a Model 600E pump system controller, a U6K universal injector and a Model 991 photodiode-array detector. The column was reversed-phase Nova-Pak C_{18} (300 × 3.9 mm I.D.). The gradient elution conditions are given in Table 1; solvent A was water–acetic acid (98:2, v/v) and solvent B was water–methanol–acetic acid (68:30:2, v/v/v). Detection was performed by scanning from 210 to 400 nm with an acquisition speed of 1 s. Spectra were recorded after subtracting the solvent absorption. Injections were made in duplicate.

2.3. Identification of compounds

Identification was achieved by comparing the retention times and the spectral data (obtained by DAD) with those for standards obtained from Sigma (Deisenhofen, Germany) and Aldrich (Steinheim/Albuch, Germany). *p*-Coumaric acid derivatives, for which standards were not avail-

Table 1
Mobile phase gradient composition and flow-rate (gradient curve No. 5)

Time (min)	Flow-rate (ml/min)	A (%)[a]	B (%)[a]
0	0.8	100	0
59	0.8	20	80
65	0.8	20	80

[a] A = water–acetic acid (98:2, v/v); B = water–methanol–acetic acid (68:30:2, v/v/v).

able, were previously identified by hydrolysis [18].

2.4. Statistical analysis

The data were subjected to statistical analysis utilizing the Statgraphics program (Statistical Graphics, Rockville, MD, USA).

3. Results and discussion

3.1. Identification of patulin

The chromatographic method utilized, which was initially intended for separating the highly complicated phenolic content of fruit juices, was also able to separate patulin in apple juice. It can be detected when present in concentrations below the toxic level (the limit of detection is 8.96 μg/l), even among other compounds with a higher UV response or present at higher concentrations (Fig. 2).

In other substrates, where patulin could be co-eluting with interfering compounds, the UV spectrum obtained by DAD permits the identification of the mycotoxin.

In previous work [17], we studied using the same chromatographic conditions 65 low-molecular-mass phenolic compounds that are common in vegetable substrates. The UV spectra and several spectral parameters derived from them are different from those of patulin (Table 2) as follows. The spectrum of patulin consists of a unique band (see also Fig. 3), whereas that of any of the studied low-molecular-mass phenolics presents at least two bands in the same wavelength range. The spectral band of patulin originates from overlapping of the absorption bands corresponding to two conjugated chromophoric groups, *i.e.*, the carbonyl group and the diene. The convexity interval of the band, defined as the distance (in nm) between the inflection points before and after the maximum, has a value that is near that of a carbonylic band in a phenolic compound when this band overlaps one of the bands of the aromatic ring. This confirms the overlapping of bands explained for patulin.

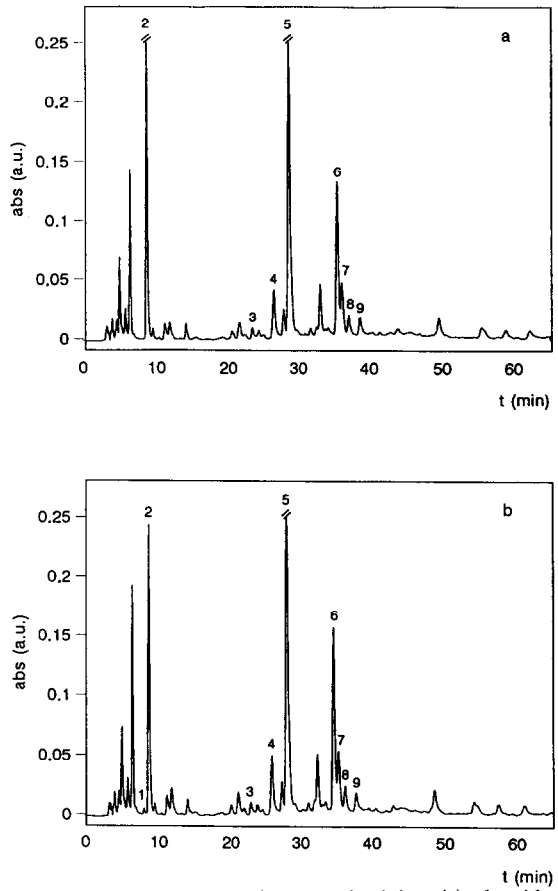

Fig. 2. Chromatograms of an apple juice (a) devoid of patulin (below the detection limit) and (b) spiked with 44.12 μg/l of patulin. Peaks: 1 = patulin; 2 = 5-hydroxymethyl-2-furaldehyde + furan-2-carboxylic acid; 3 = (+)-catechin; 4 = caffeic acid; 5 = chlorogenic acid; 6 = *p*-coumaroylquinic acid; 7 = *p*-coumaric acid; 8 = *p*-coumaroylglucose; 9 = (−)-epicatechin.

Other spectral parameters studied in the previous work [17], such as absorbance ratios at different wavelengths and the positions of the maxima in the second-order derivative spectrum, are also useful for confirmation of the identity of patulin.

3.2. Recovery of patulin

As the extraction of a compound may be affected by the other substances present in the sample, we calculated the recovery of patulin from an apple juice spiked with patulin to final concentrations of 89.46, 44.12, 17.86 and 8.96

Table 2
Values of the chromatographic parameters obtained with diode-array detection

Parameter	Patulin		5-Hydroxymethyl-2-furaldehyde		Furan-2-carboxylic acid	
Retention time (min)	8.0		8.6		8.9	
Absorbance maxima (nm)	275.4		231.5	282.2	229.8	252.3
Convexity intervals (nm)	36		–	30.6	–	26.0
260/320 nm absorbance ratio	13.6		15.7		390.6	
270/300 nm absorbance ratio	3.0		1.46		14.8	
Absorbance maxima (nm) in the second-derivative spectrum	239.3	309.5	249.7	309.5	232.8	275.7

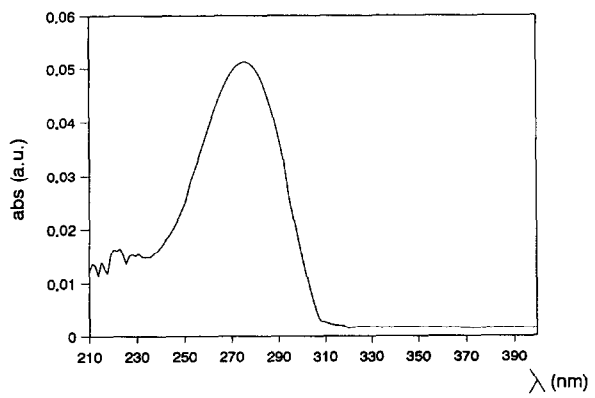

Fig. 3. UV spectrum of patulin.

μg/l. The determination of patulin was performed at 280 nm by the external standard method. A calibration graph with a higher correlation coefficient was obtained when working with peak area ($r = 0.99898$) than with peak height ($r = 0.96111$).

The recovery of patulin (Table 3) decreases with increasing concentration, ranging from 82.3% to 42.0% (because at very low concentrations the partition coefficient does not show a linear behaviour). The value of 8.96 μg/l can be considered as the detection limit of the method as lower concentrations do not allow a proper definition of the spectrum. This value is close to those reported in the literature.

Table 3
Recovery of patulin added to commercial apple juices

Concentration present (μg/l)	Concentration found (μg/l)	Recovery (%)	S.D. (%)
89.46	73.81	82.3	5.6
44.12	33.11	75.0	5.5
17.86	9.93	55.6	2.2
8.96	3.76	42.0	7.7

Results based on three replicate analyses.

3.3. Applicability

The chromatograms of a commercial apple juice without patulin and spiked with 44.12 μg/l (below the concentration considered critical by legislators) are shown in Fig. 2. Peak 1 corresponds to patulin and peaks 3–9 to phenolic compounds usually found in apples.

The nearest peak to that of patulin, peak 2, is not a phenolic compound, but appears in industrially produced fruit juices. It has been identified as a mixture of 5-hydroxymethyl-2-furaldehyde and furan-2-carboxylic acid. DAD permitted the identification of these compounds, their separate spectra to be obtained and the spectral parameters mentioned above to be acquired (Table 2). Although those compounds are related to patulin, also having a furan ring, they can be distinguished from it by the presence of two bands in their spectra and by the values of the spectral parameters.

4. Conclusions

The usefulness of DAD in the identification of a compound such as patulin in apple juice, in the

presence of other compounds occurring at very different concentrations, and having similar or dissimilar chemical attributes, has been demonstrated. These compounds can be naturally occurring in the fruits (phenolics), or can be formed during the industrial treatment applied to juices (furfurals).

The detection limit of patulin is slightly less than 8.96 μg/l, with a recovery of 42%, which increases to 75% for the legally critical concentration of 50 μg/l and above. The chromatographic system allows the determination of patulin very rapidly (less than 9 min) if it is the unique purpose of the analysis; the complete run allows the phenolic composition of the juice to be studied simultaneously, study for authentication or quality control purposes.

5. Acknowledgements

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