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Rapid reversed-phase liquid chromatographic determination of patulin in apple juice

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Abstract

A rapid, simple and economical method using a limited amount of organic solvent is described for the determination of patulin in apple juice. The sample was extracted with ethyl acetate and the extract was cleaned up by extraction with sodium carbonate solution. Patulin was then determined by reversed-phase liquid chromatography using a MicroPak C_{18} column and a variable-wavelength UV–Vis detector set at 276 nm. Patulin and 5-hydroxymethylfurfural were completely resolved by using water–acetonitrile (99:1, v/v) as the mobile phase at a flow-rate of 1.0 ml/min. The detection limit was <5 μg/l and the recovery was 98%.

Keywords: Apple juice; Patulin; Mycotoxins

1. Introduction

Patulin, 4-hydroxy-4*H*-furol[3,2-*c*]pyran-2(6*H*)-one, is a mycotoxin produced by several species of *Penicillium* and *Aspergillus*. It is soluble in a wide variety of organic solvents and also in water [1–6]).

Patulin has mainly been found in apples and apple products such as juice, juice concentrate, jam and confectionary. It has also been determined in pears, apricots, peaches, tomatoes and oranges and in products derived from these fruits [2,5]. Levels of patulin in fruit products can be reduced by removing rotten tissue from the fruit. Patulin can therefore be used as an indicator of the quality of processed apple juice and fruit products, since appreciable concentrations of the toxin remain in food after processing [7]. Owing to its toxicity, health authorities in many countries regard patulin contamination of foods as a problem and have set a maximum permitted concentration (MPC) of 50 μg/l for patulin in apple juice appropriately diluted for consumption [4,6,7]. Therefore, a rapid and reliable method for the determination of patulin is important to manufacturers and governments interested in monitoring the quality of apple juice and concentrate [4].

Many methods have been developed for measuring patulin in apple juice. These include methods based on thin-layer chromatography (TLC) and more recently high-performance liquid chromatography (HPLC) [8–14]. However, poor resolution of patulin from co-extracted interfering substances, especially 5-hydroxymethylfurfural (5-HMF), is an important problem that must be considered in both TLC and HPLC methods. TLC methods that provide qualitative or semi-quantitative results are considerably expensive and time consuming owing

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to the use of large amounts of organic solvents and complicated clean-up procedures. In addition, their sensitivity is poor with the limit of detection generally given as 20–25 μg/l [9,12,13]. HPLC methods lead to an improvement in sensitivity. However, clean-up procedures in some HPLC methods are similar to those in TLC, using column chromatography [8,15] or clean-up cartridges, which are not convenient [4–5].

In this study, a rapid, simple and economical method using limited amounts of organic solvents was developed for the determination of patulin in apple juice.

2. Experimental

2.1. Materials

Commercial apple juice samples were obtained from local markets in Ankara. Apple juice samples provided from an AOAC–IUPAC collaborative study for patulin in apple juice were also analysed. Each sample was extracted in duplicate with duplicate injections into the column.

2.2. Apparatus

High-performance liquid chromatograph

A Varian Model 9010 liquid chromatograph was used. It was equipped with a Rheodyne Model 7161 six-way injector with a 10-μl loop and a Varian Model 9050 variable-wavelength UV–Vis detector set at 276 nm. The chromatograms were recorded by using a Varian Model 4400 integrator with a chart speed of 1.0 cm/min.

Column

The analytical column (150 × 4 mm I.D.) was made of stainless steel and was packed with 5-μm C_{18} stationary phase and operated at ambient temperature. It was protected by a microparticulate (C_{18}) guard column (40 × 4 mm I.D.).

Mobile phase

Water–acetonitrile (99:1, v/v) was used at a flow rate of 1.0 ml/min. It was filtered through a 0.45-μm regenerated cellulose acetate membrane and degassed ultrasioncally just before HPLC analysis.

2.3. Chemicals

Ethyl acetate (extra pure), acetonitrile (HPLC grade), acetic acid (extra pure), sodium carbonate (reagent grade) and anhydrous sodium sulfate (reagent grade) were obtained from Merck (Darmstadt, Germany). Water used in all the experiments was doubly distilled and deionized.

A stock standard solution of patulin was prepared by dissolving 5 mg of pure crystalline patulin (Merck) in 25 ml of ethyl acetate. A 100-μl volume of this solution was transferred into a 10-ml volumetric flask and evaporated just to dryness under a stream of nitrogen at room temperature. The residue was immediately dissolved in 10 ml of water (pH 4.0) acidified with acetic acid. Working standard solutions were prepared by appropriate dilution of this solution with water (pH 4.0).

A stock standard solution of 5-HMF was prepared by dissolving 5 mg of 5-HMF in 25 ml of ethyl acetate. Sodium carbonate solution (1.5%, w/w) was prepared in doubly distilled water.

2.4. Extraction procedure

A 5-ml volume of single-strength apple juice was extracted twice with 10 ml of ethyl acetate by shaking vigorously for 1 min using a vortex mixer. The organic phases were combined and extracted with 2 ml of 1.5% sodium carbonate solution by shaking for 1 min. The phases were allowed to separate and the aqueous phase was immediately extracted with 5 ml of ethyl acetate by shaking for 1 min. The combined organic phases were dried over 2.5 g of anhydrous sodium sulfate. Subsequently, the dried extract was filtered through a black band filter-paper to remove the remaining particles of anhydrous sodium sulfate. A 2-ml excess of ethyl acetate was added to wash the filter cake layer and the

filtrate obtained was combined with the filtered extract. Then the extract was evaporated just to dryness in a water bath at 40°C under a gentle stream of nitrogen. The residue was immediately dissolved in 500 μl of water (pH 4.0) and 10 μl of this solution were injected into the column. The final solutions were kept in deep-freezer until the chromatographic measurements.

2.5. Calculation of results

The amount of patulin in the final solution was determined by using a calibration graph of concentration vs. peak height and expressed as μg/ml. The patulin content (C) of the apple juice was found by using the equation

$$C\ (\mathrm{mg/l}) = \frac{C_{\mathrm{spl}} \cdot V \cdot 1000}{m}$$

where C_{spl} is the concentration of patulin in the final solution (μg/ml), V is the total volume of the final solution (ml) and m is the volume of apple juice taken for extraction (ml).

2.6. Recovery

Apple juices containing known amounts of patulin were spiked with 25, 50, 100, 150 and 200 μg/l of patulin to determine the recovery of the extraction procedure.

2.7. Performance test

Volumes of 250 μl of the solutions containing both patulin and 5-HMF at a concentration of 2 μg/ml were transferred into a 1-ml volumetric flask and evaporated to dryness under a stream of nitrogen. The residue was dissolved in 1 ml of water (pH 4.0) and 10 μl were injected into the column to test the column performance.

3. Results and discussion

3.1. Separation of patulin from 5-HMF

The mobile phase and column used in the HPLC determination were selected to yield the maximum separation of patulin from interfering substances in apple juice, especially 5-HMF. The detection wavelength was set at 276 nm, corresponding to the maximum UV absorption, as used by Geipel et al. [8]. Neukom et al. [3] also proposed the use of ISO standard method No. 8128, Part 2 [13]. Although the separation of patulin and 5-HMF has been achieved previously using water [14] and tetrahydrofuran–water (1:99) [4,5] as the mobile phase, it was not possible to resolve patulin from 5-HMF in this study using water alone. Additionally, tetrahydrofuran is not as convenient a solvent as acetonitrile in reversed-phase HPLC applications. Acetonitrile–water (10:90, v/v) proposed by ISO [13] was also found to be inadequate owing to poor resolution. A high degree of separation and a lower consumption of acetonitrile could be achieved when the proportion of acetonitrile in the mobile phase was reduced from 10% to 1%. Fig. 1 illustrates the separation of patulin from 5-HMF on a MikroPak C_{18} column using water–acetonitrile (99:1, v/v) as the mobile phase at a flow-rate of 1.0 ml/min. Patulin and 5-HMF

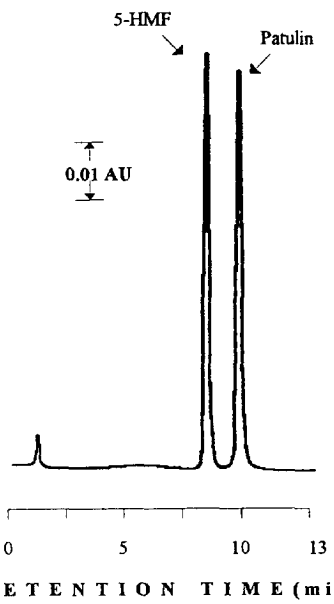

Fig. 1. Separation of patulin and 5-HMF on a MikroPak C_{18} column. Chromatographic conditions: column, C_{18} (5 μm), 15 cm × 4 mm I.D., protected by a microparticulate guard column (4 cm × 4 mm I.D.); mobile phase, water–acetonitrile (99:1, v/v); flow-rate, 1.0 ml/min; detection wavelength, 276 nm; temperature, ambient; injection volume, 10 μl.

were completely resolved from each other in an elution time of ca. 13 min. The correlation between the concentration and the corresponding peak height was determined to be linear for the concentration range 0.1–2 ng/μl ($r = 0.9997$, $n = 5$).

3.2. Recovery

Fig. 2 illustrates the chromatograms of apple juices both unspiked (control) and spiked with 25 and 50 μg/l of patulin. The recovery of patulin in apple juice was 94% or higher for added levels of 25, 50, 100, and 200 μg/l (Table 1). The recovery obtained in this study is higher than those recorded in many methods using HPLC [4–6,8]. It is thought that this is due to the use of a double extraction with ethyl acetate using a sample-to-solvent volume ratio of 1:2, as explained by Prieta et al. [5].

Clean-up by extraction with 1.5% sodium carbonate solution has been found to be applicable in many studies, indicating the necessity for fast extraction owing to the instability of patulin in alkaline solution [3,6,10,13]. Here, the ethyl acetate extract was also cleaned up by extracting with 1.5% sodium carbonate solution. The procedure is simple, rapid and economical when compared with clean-up using adsorption chromatography or clean-up cartridges. It was found to be very effective for removing most of the co-extractives from apple juice. Therefore, the use of either adsorption chromatography on silica gel [8,15] or prepacked clean-up cartridges [4,5] does not seem to be necessary.

Table 1
Recovery of patulin from spiked apple juices

Spiking level (μg/l)	Recovered (μg/l)	Recovery (%)	S.D. (%) ($n = 4$)
25	24.5	98.3	0.55
50	62.5	125.5	1.16
100	95.7	95.7	0.84
150	157.0	104.6	1.40
200	188.4	94.2	0.68

The limit of detection achieved meets the requirements for quality control purposes taking into account the MPC level of 50 μg/l well established in many countries and also recom-

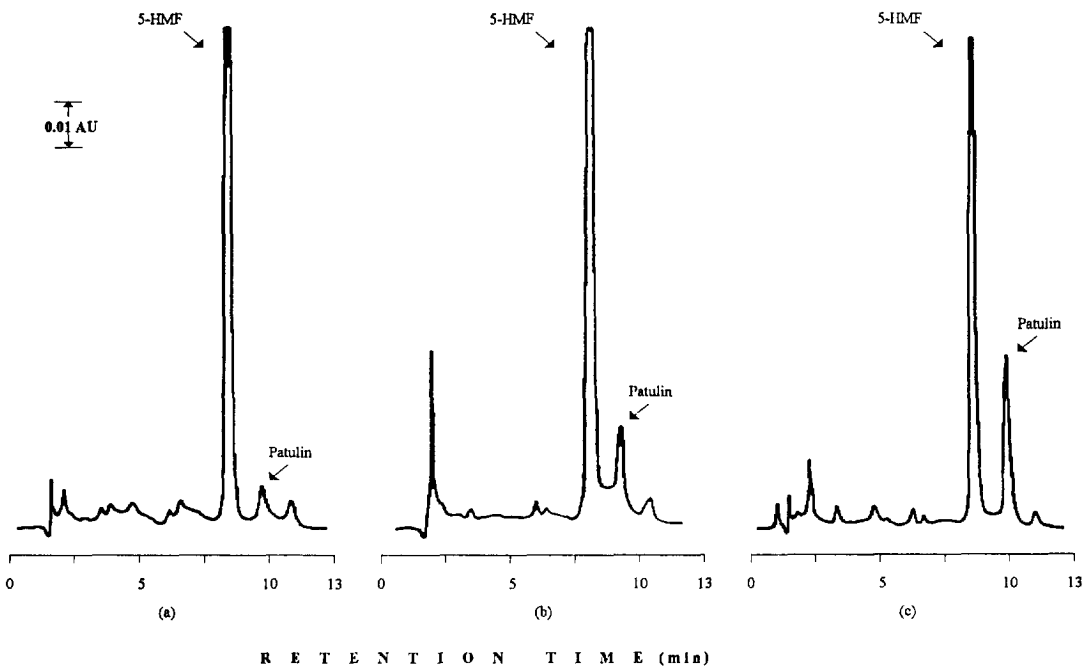

Fig. 2. Chromatograms of apple juice samples, unspiked (control) and spiked with different levels of patulin: (a) 18 μg/ml (control); (b) 43 μg/ml (control + 25 μg/ml); (c) 68 μg/ml (control + 50 μg/ml). Chromatographic conditions as in Fig. 1.

mended by WHO. Under the stated conditions, as little as 4 μg/l of patulin in apple juice could be detected. The chromatogram of apple juice containing 4 μg/l of patulin is shown in Fig. 3.

The applicability of this method was tested by analysing apple juice samples containing unknown amounts of patulin, provided from an AOAC–IUPAC collaborative study on the determination of patulin in apple juice by HPLC. The results obtained were found to be very close to the original patulin levels, except for one sample containing 5 μg/l of patulin (Table 2).

A limited number of commercial apple juice samples obtained periodically from local markets in Ankara were also analysed to monitor the patulin contamination levels. Patulin was detected in 95% of all commercial apple juice samples (Table 3). The contamination levels of fifteen commercial samples were found to be higher than 20 μg/l, but only two of samples contained patulin at levels higher than 50 μg/l, the MPC level well established in many countries.

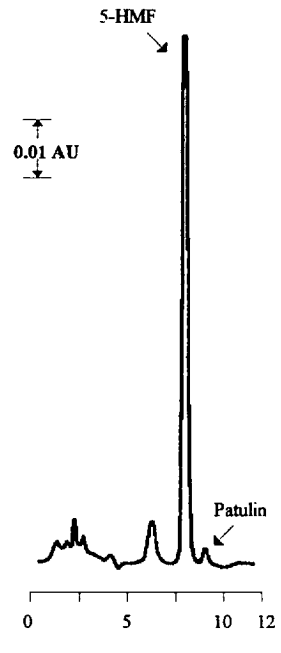

Fig. 3. Chromatogram of apple juice sample contaminated with 4 μg/l of patulin. Chromatographic conditions as in Fig. 1.

Table 2
Patulin levels found in the samples provided from an AOAC–IUPAC collaborative study

Patulin level in unknown sample (μg/l)	Patulin level found (μg/l)	*n*	S.D. (μg/l)
5	15.5	2	0.59
20	23.5	2	1.03
50	47.1	8	1.09
100	125.0	2	0.84
200	188.5	4	3.48

Table 3
Patulin levels in commercial apple juices obtained from Ankara

Patulin level (μg/l)	No. of samples
<20 μg/l	5
20–50 μg/l	13
>50 μg/l	2

Samples obtained in 1993 and analysed in 1994.

In summary, the method described here uses an extraction and clean-up procedure that is very similar to those described by Möller and Joseffson [14], Forbito and Babsky [6] and Neukom et al. [3], but it gives better recoveries. The chromatographic conditions, the mobile phase and the column, are suitable for the determination of patulin in apple juice with good resolution and reproducible results. The sensitivity achieved is sufficient to allow the detection of 4 μg/l of patulin, thereby allowing the determination of the patulin levels in apple juice recommended by the WHO as a safety margin. Also, the method is suitable for both goverment agencies and manufacturers interested in monitoring the quality of commercial apple juice.

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