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# Determination of Patulin in Apple Juice Produced in Isparta, Turkey by HPLC with Diode Array Detection

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## ABSTRACT

In this study patulin was determined by HPLC on a reversed phase C18 column with UV detection at 276 nm. Patulin was extracted with ethyl acetate and then was cleaned up by extraction with sodium carbonate solution. The mobile phase and the column used in the HPLC determination were selected to separate patulin from the interfering substances in apple juice, especially 5-hydroxymethylfurfural (HMF). The linear regression coefficient of the standard curve (y = 327x – 292) for patulin at concentrations ranging from 15 to 200 $\mu$g/L was 0.9998 (N = 6). The results were corrected for the recovery percentage. The method is rapid and convenient for the determination of patulin in apple juice.

Key words: patulin, HPLC, fruit juice

## INTRODUCTION

Patulin, 4-hydroxy-4H-fural[3,2-c]pyran-2(6H)-one, is acutely toxic[(1,2)], carcinogenic, teratogenic and mutagenic[(3,4,5)]. Patulin has been found to be a natural contaminant of processed apple products and its presence was suggested to be indicative of the quality of the fruit used in production[(6)]. It is particularly produced by the apple-rotting fungus, penicillium expansum, thus being of toxicological concern in apple products such as apple juice[(7)]. The maximum permitted concentration in foodstuff has been set at 50 $\mu$g/L by the World Health Organization (WHO)[(8)]. HMF is an important quality criterion in fruit juices and a known indicator of temperature treatment of different food products. HMF is formed as a result of dehydration of sugars, particularly under acidic and high temperature conditions[(9)]. HMF and patulin have similar chromatographic properties owing to their chemical structures and therefore, HMF is observed as the main interference during the liquid chromatographic analysis of patulin[(10,11,12)].

A method for patulin determination 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. This method has undergone slight modifications, but forms the basis of currently recommended HPLC methods. It was slightly modified by Möller and Joseffsson to enable the clean up to be performed on a micro scale, whereas Forbito and Babsky published a modification to the HPLC mobile phase for improved resolution of the patulin. In its original form, the method formed part of a collaborative study in which the performances of two reversed-phase HPLC methods for the determination of patulin in apple juice were evaluated in 12 laboratories from 10 countries. Patulin is a low-molecular-mass, polar molecule which is only retained on reversed-phase HPLC columns by the use of common mobile phases, i.e. 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[(13)].

In this study, patulin was determined in apple juice produced from apples collected from Isparta region of Turkey using HPLC technique. The effect of HMF interference was also investigated.

## MATERIALS AND METHODS

### I. *Chemicals*

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

### II. *Sample Preparation*

Eight mL of apple concentrate (having 70˚Brix) was diluted to 50 mL, so Brix became 11.2˚ which corresponds to single strength apple juice[(14)]. An aliquot (5 mL) of clear apple juice was sequentially extracted with two 10-mL portion of ethylacetate in a separator funnel for 2 min. The aqueous phase was discarded. The pooled ethyl-

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acetate fractions were washed in the same separator funnel with 2 mL of aqueous $Na_2CO_3$ solution (1.5%, w/v) to remove phenolic acids. This step was completed within 1-2 minutes, since patulin is unstable under alkaline conditions. Sodium carbonate treated solution was mixed with 10 mL of ethylacetate in the separator funnel. Two grams of anhydrous $Na_2SO_4$ was added to the ethylacetate fraction as a drying agent. After immediate swirling, the dried extract was filtered through a black band filter paper to remove the remaining particles of anhydrous $Na_2SO_4$. Two mL of excess ethylacetate was added to wash the filter cake layer and the filtrate obtained was combined with the filtered extract. The filtrate was acidified by 5 drops of acetic acid. The organic solvent was removed by rotary evaporation in a water bath at 40 ± 1°C under a gentle stream of nitrogen. The residue was redissolved in 500 μL of water that had been acidified by acetic acid (pH 4.0) and 20 μL of this solution was injected into the column.

III. *Stock Solution*

A stock standard solution of patulin was prepared by dissolving 5 mg of pure crystalline patulin (Sigma) in ethylacetate of pH 4.0 and made up to 25 mL with the same ethylacetate solution. Stock solution (with concentrations of 200 mg/L) was kept in a deep freezer (at –18°C).

IV. *HPLC Apparatus*

The HPLC analysis was carried out on a Shimadzu model LC-VP HPLC system with model LC-VP software. It was equipped with control unit (SCL 10 AVP), pump (LC-10 ADVP), detector (SPD-M 10A VP photo diode array), auto sampler (SIL-10 AD), column furnace (CTO 10 AVP) and gas remove unit (DGU 14 A) (Kyoto, Japan).

V. *Column*

The analytical column (100 × 4.6 mm i.d.) obtained from ICT-Handels-GmbH (BAD Hamburg, Germany) was made of stainless steel and was packed with 3 μm Astec C18 stationary phase. The other one (250 × 4.6 mm i.d.) obtained from Phenomenex (Torrance, CA, USA) was packed with 5 μm Luna C18 stationary phase.

VI. *Mobiles Phases*

Four different mobile phases were used.

Mobile phase 1: 25 mL of acetonitrile and 0.0564 g of tetrabutylammonium bromide were mixed and made 250 mL with pH 4.0 acetic acid solution, so the concentration of ion pair producer became 0.7 mM. Nucleosil Macherey Nagel (250 × 4.6 mm i.d. 5 μm) column (Macherey - Nagel GmbH & Co. KG, Gutenberg, Germany) was used. Flow rate was 1.5 mL/min.

Mobile phase 2: methanol/water (9/91, v/v) with the same column as mobile phase 1. Flow rate was 0.8 mL/min.

Mobile phase 3: acetonitrile/water (5/95, v/v) with Luna C18 (100 × 4.6 mm i.d. 3 μm) column. Flow rate was 1.0 mL/min.

Mobile phase 4: acetonitrile: water (5/95, v/v) with Astec C18 (100 × 4.6 mm i.d. 3 μm) spherical column. Flow rate was 0.8 mL/min.

VII. *Chromatographic Procedure*

A flow rate of 0.8 mL/min was selected for all chromatographic separations. The separation column was equilibrated with mobile phase until the baseline was stabilized. Sample injections were made at this point. The time required by the mobile phase to convey a solute from the point of injection onto the stationary phase, through the stationary phase, and to the detector is defined as the retention time. The dead time, $t_o$, was established for mobile phase tested by injection of a acetonitrile solution in water monitoring the eluate at 190 nm. The capacity factor, k, is a measure of the number of column volumes required to retain a compound. It is defined mathematically as:[15]

where $t_r$ = is the retention volume of a nonretained peak.

$$k = \frac{t_r - t_o}{t_o}$$

The system dead time, $t_o$, used to calculate capacity factor k, was measured by injecting acetonitrile solution into the system. An average of at least three replicates was used to do all the calculations.

## RESULTS AND DISCUSSION

In this study, the juices obtained from the best quality apples collected throughout the Isparta region were analyzed. The batches of apples obtained from the apples raised between August and November, which is the harvest season in our city, were analyzed. Extractions were done for five different batches of apple juice concentrate, each of which was taken from 7 kg of apple. It is possible to assume that the apple juices analyzed can represent the entire Isparta region. Before the analysis of apple juice samples, the standard mixture of patulin and HMF were subjected to a series of chromatographic runs in order to establish optimum conditions for identificating these two components in the mixture.

I. *Separation of HMF and Patulin*

Results showed that acetonitrile in mobile phase mixture at 5% (v/v) provided better separation in C18 column with the flow rate of 0.8 mL/min (Mobile phase 4) (Figure 1).

Retention times of HMF and patulin were found as 4.72 and 6.16 min, respectively (Table 1).

The $t_o$ value of acetonitrile was found to be 1.95 min

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**Table 1.** Retention times of HMF and patulin under different conditions

| Mobile phase | Retention time (min) | |
|---|---|---|
| | HMF | Patulin |
| 1 | 3.61 | 4.72 |
| 2 | 12.51 | 13.81 |
| 3 | 7.81 | 9.62 |
| 4 | 4.72 | 6.16 |

**Table 2.** Analytical results for patulin in apple juice samples

| Sample No. | Patulin (ppb) | $R^2$ | CV % | Recovery % |
|---|---|---|---|---|
| 1 | 69.62 ± 0.31 | 0.9993 | 3.39 | 95 |
| 2 | 43.56 ± 3.06 | 0.9999 | 2.61 | 105 |
| 3 | 40.62 ± 9.37 | 0.9995 | 2.82 | 76 |
| 4 | 46.87 ± 3.75 | 0.9999 | 4.63 | 74 |
| 5 | 47.06 ± 9.37 | 0.9995 | 4.28 | 98 |

at 190 nm. From this value, capacity factors (k values) of HMF and Patulin were obtained as 1.42 and 2.16 respectively. From the division of the value for patulin to the one for HMF was found 1.52 as the selectivity (α value).

II. *Separation Efficiency*

Using six different concentrated solutions of patulin (in the range of 15 to 200 ppb) chromatographic determination was carried out with mobile phase 4, C18 column and flow rate of 0.8 mL/min. Linearity was observed with concentration up to 200 ppb.

Standard curves (y = 327x – 292, n = 6) with a linear regression coefficient ($R^2$) of 0.9998 for normal calibration method and (y = 0.95x + 105.46, n = 3) with linear regression coefficient of 0.9995 for standard addition method were obtained. In this equation, y represents peak area (mAU) and x represents concentration (ppb) (Figure 2). RSDs were 2.06 and 0.0078% for normal calibration method and standard addition method, respectively.

III. *Recovery*

Apple juice samples containing known amounts of patulin were spiked with the different levels of patulin to determine the recovery of the extraction procedure.

As shown in Table 2, the recovery values of patulin in apple juice were in the range of 74 to 105%. Lower percent recovery namely, 74 and 76%, might result from several reasons such as insufficient separation of organic phase in the separator funnel, contamination, prolonged evaporation under nitrogen of high flow rates and insufficient extraction time.

Using the optimum chromatographic conditions, analysis of apple juices was carried out. Results are given in Table 2 and Figure 3 In Figure 3, a peak appeared at around 10 min and retention time could not be identified.

IV. *Sensitivity*

Patulin levels of four different groups of apple juices did not exceed the maximum permitted level of 50 μg/L except one group which had patulin concentration of about 70 μg/L. In a previous study done in Turkey, patulin was detected in a number of samples at concentrations ranging from 7 to 376 μg/L[14]. In this study, apple juice concen-

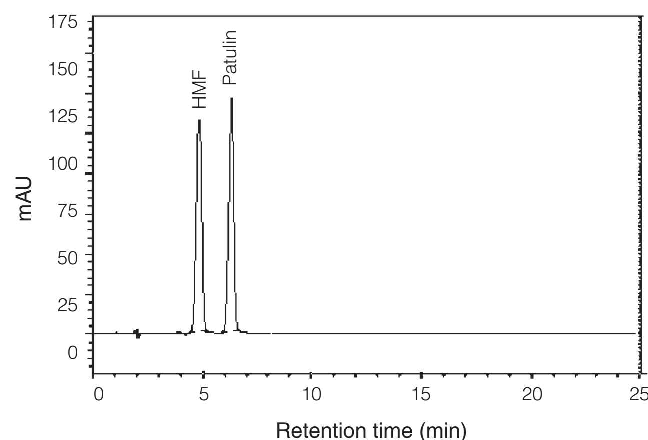


**Figure 1.** Chromatogram of HMF and patulin mixture standard. Mobile phase: acetonitrile/water (5/95), column: C18 (100 × 4.6 mm i.d. 3μm).

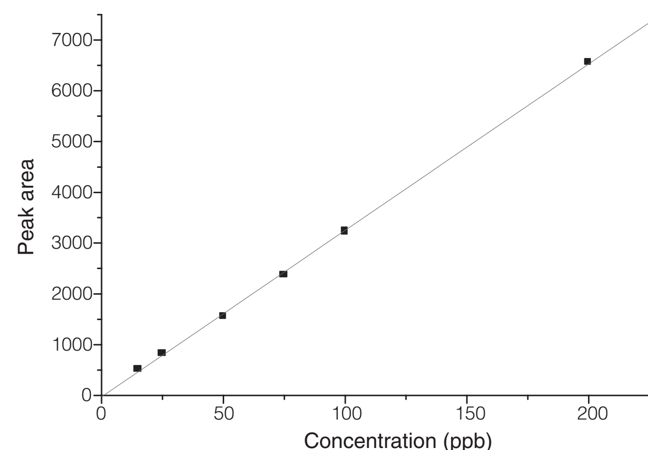


**Figure 2.** Variation of peak area against concentration of patulin.

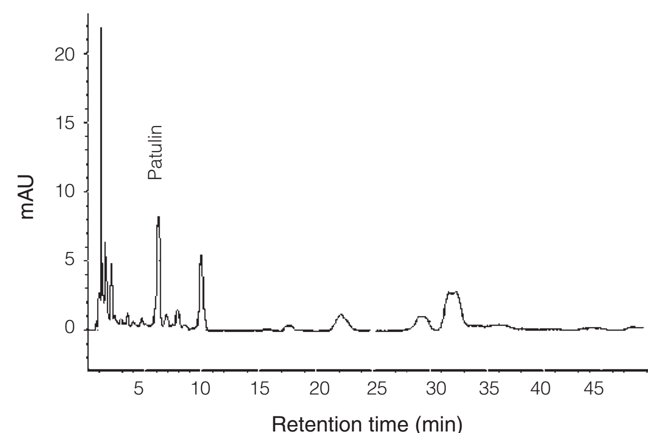


**Figure 3.** Chromatogram of patulin in apple juice. Mobile phase: acetonitrile: water (5/95), column: Luna C18 (100 × 4.6 mm i.d. 3 μm).

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trates were collected from three different companies in Turkey for the same processing season and patulin levels exceeded 50 $\mu$g/L in 46 % of all samples analyzed.

In this study, only Isparta region was taken into account and patulin levels of apple juices did not exceed 50 $\mu$g/L, except one group which was not too much greater than the permitted level (being about 70 $\mu$g/L).

It can be concluded that the amounts of patulin in most apple juice concentrates produced in Isparta region are acceptable, with a significant proportion of the products not exceeding 50 $\mu$g/L limit for apple juice set by the World Health Organization.

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