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Short communication

Simultaneous determination of 5-hydroxymethylfurfural and patulin in apple juice by reversed-phase liquid chromatography

Vural Gökmen*, Jale Acar

Department of Food Engineering, Hacettepe University, 06532 Beytepe, Ankara, Turkey

Abstract

A rapid, simple and economical method was described for the simultaneous determination of 5-hydroxymethylfurfural (HMF) and patulin in apple juice. The sample was extracted with ethyl acetate and the extract was then cleaned up by extraction with a sodium carbonate solution. Then HMF and patulin were determined by reversed-phase liquid chromatography using a C_{18} column and a photodiode array detector. HMF and patulin could be completely resolved by using the mixture water–acetonitrile (99:1, v/v) as the mobile phase with a flow rate of 1.0 ml/min. Mean recoveries of HMF ranged from 86% to 100% with an overall mean of 94%, that of patulin ranged from 94% to 125% with an overall mean of 103%, for different spiking levels. The limits of detection for HMF and patulin in apple juice were found to be <0.01 mg/l and <5 μg/l, respectively.

Keywords: Food analysis; Fruit juices; Hydroxymethylfurfural; Patulin; Mycotoxins

1. Introduction

Both 5-hydroxymethylfurfural (HMF) and patulin are important quality criteria in fruit juices. The presence of HMF is considered as an indication of quality deterioration. It is formed as a result of dehydration of ketopentoses, particularly in acidic or high-temperature environments [1,2]. This chemical conversion has been reported to occur during the storage of orange juices where the concentration of HMF increased, as the storage temperature was increased [3]. The presence of HMF in stored apple juices was also reported [4,5]. Sulc has reported that evaporation carried out at higher temperatures to concentrate strawberry juice produced non-enzymatic browning and HMF in juice where HMF contents rose during storage [6]. The International Federation of Fruit Juice Processors (IFFJP) recommends a maximum concentration of 5–10 mg/l HMF in fruit juices and 25 mg/kg in concentrates [7].

Patulin is a mycotoxin mainly found in apples and apple products, and has become one of the most important quality criteria for apple juice. Patulin is formed in apples decayed by certain genus of molds, particularly *Penicillium expansum*, easily transfers into apple juice during processing owing to its solubility in water [8–10]. It is very stable to heat in acidic medium as in apple juice, therefore noticeable amount of patulin still remains in juice after processing [11,12]. Carcinogenic, teratogenic and mutagenic effects of patulin have been reported [13,14]. A maximum permitted concentration has been set at 50 $\mu g\ l^{-1} \cdot kg$ in foodstuffs by the World Health Organisation (WHO) [15].

Various analytical methods have been reported for the determination of HMF and patulin, separately in fruit juices [16–22]. HMF and patulin exhibits similar chromatographic properties owing to their

*Corresponding author.

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5-Hydroxymethyl-2-furaldehyde **HMF**

4-Hydroxy-4H-furol[3,2-c]pyran-2(6H)-one **Patulin**

Fig. 1. Chemical structures of HMF and patulin.

chemical structures (Fig. 1), and therefore, HMF is appeared as the most encountered interference during the liquid chromatographic analysis of patulin [23]. This led us to investigate the possibility of simultaneous determination of these constituents in apple juice.

2. Experimental

2.1. Material

Apple juice concentrate samples were obtained from the manufacturers in Turkey. Samples were diluted into an approximate percentage soluble solid content (Brix) of 11.2% by doubly distilled water. Each sample was extracted in duplicate with duplicate injections into the column.

2.2. Apparatus

2.2.1. High-performance liquid chromatography

A model SpectraSystem P4000 liquid chromatograph (Thermo Separation Products, San Jose, CA, USA) was used. It was equipped with a model 7161 six-way injector with a 20 μl sample loop (Rheodyne, Cotati, CA, USA), a model SpectraFocus forward optical scanning detector (Thermo Separation Products) operated at a wavelength interval of 250–300 nm, and a helium degassing system. The chromatograms were recorded by using a PC1000 version 3.0 data acquisition system.

2.2.2. Column

The analytical column (150×4 mm I.D.) obtained from Phenomenex (Torrance, CA, USA) was made of stainless steel and was packed with 5 μm C_{18} stationary phase and operated at ambient temperature.

2.2.3. 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 (Millipore, Bedford, MA, USA) and on-line degassed by a gentle stream of helium.

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 deionised.

Stock solution of HMF was prepared by dissolving 1 g of pure HMF (Merck) in 100 ml of ethyl acetate, then diluting this solution 1:50 (v/v) by ethyl acetate to obtain a final HMF concentration of 0.2 mg/ml. Stock solution of patulin was also prepared at a concentration of 0.2 mg/ml by dissolving 5 mg of pure crystalline patulin (Merck) in 25 ml of ethyl acetate. A 100 μl volume of these stock solutions was transferred into a 10 ml volumetric flask separately and evaporated just to dryness under a gentle stream of nitrogen at room temperature. The residues were immediately dissolved in 10 ml of water (pH 4.0) acidified with acetic acid. Working standard solutions were prepared by appropriate dilution of these solutions with water (pH 4.0).

Sodium carbonate solution (1.5%, w/w) was prepared in doubly distilled water.

2.4. Analytical procedure

The extraction was carried out according to the method described by us elsewhere for the determination of patulin only in apple juice [23]. A 5 ml volume of apple juice (approximately 11.2% Brix) 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 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 at a total volume of 25 ml were dried over 2.5 g of anhydrous sodium sulfate. Subsequently, the dried extract was filtered through a S&S No.589[3] black band filter paper (Schleicher and Schuell, Germany) 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 20 μl of this solution was injected into the column. The final solutions were kept frozen (−18°C) until the chromatographic measurements.

3. Calculation of results

The amounts of HMF and patulin in the final solution were determined by using a calibration graph of concentration vs. peak area and expressed as μg/ml. The respective concentrations of HMF and patulin in apple juice were found by using the following equations;

$$C_{\mathrm{HMF}}\,(mg/l) = \frac{C^{*}_{\mathrm{HMF}} V}{m}$$

$$C_{\mathrm{Patulin}}\,(\mu g/l) = \frac{C^{*}_{\mathrm{Patulin}} V 1000}{m}$$

where C^{*}_{HMF} and C^{*}_{Patulin} are the concentrations of HMF and patulin in the final solution (μg/ml), V is the total volume of the final solution (ml), and m is the volume of diluted apple juice taken for extraction.

The results calculated were corrected for the brix of sample since all quality criteria of apple juice are given for a Brix value of 11.2% in trade.

3.1. Recovery

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

3.2. Applicability

Various apple juice concentrates produced in Turkey were analysed in order to test the applicability of method. The results were compared with standard colorimetric toluidine–barbituric acid method [7]. Relatively low and high HMF concentration effects on the method's applicability were also examined.

4. Results and discussion

4.1. Separation efficiency

The standard mixture of HMF and patulin was subjected to a series of chromatographic runs in order to establish optimum condition for separation in C_{18} column. As stated earlier, water used alone as the mobile phase was not found sufficient to resolve HMF and patulin. Including acetonitrile in the mobile phase mixture at 1% (v/v) resulted a better separation owing to faster elution. However, further increase in acetonitrile composition did not cause noticeable improvement, but adversely affected the separation. Although this result is convenient with our previous findings, it should be noted that the mobile phase composition needs to be optimised for individual conditions. Fig. 2 illustrates the separation of pure HMF and patulin on a Phenomenex C_{18} column using water–acetonitrile (99:1, v/v) as the mobile phase at a flow rate of 1.0 ml/min. Fig. 3 also illustrates the apple juice chromatogram in

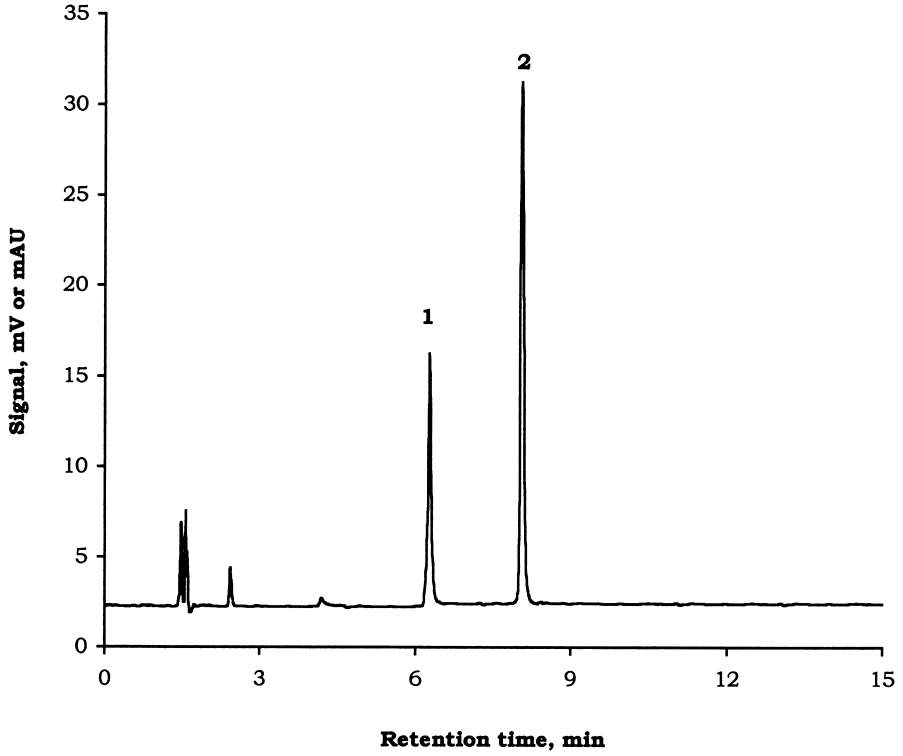

Fig. 2. Separation of HMF and patulin on a C_{18} column, (1) HMF, (2) Patulin (respective concentrations are 1 μg/ml for both HMF and patulin in the standard mixture).

which all co-extractives were resolved from each other under the same chromatographic conditions.

Correlation coefficients based on the concentration (μg/ml) versus peak area (mAU) were 0.997 ($y=40517x+1162$, $n=5$) and 0.998 ($y=115284x+882$, $n=5$) for HMF and patulin, respectively. Retention times of HMF and patulin were approximately 6.3 and 8.1 min, respectively. RSDs were 0.49 and 0.65% for HMF and patulin, respectively, indicating good retention time reproducibility.

4.2. Recovery

As stated earlier, the recovery of patulin in apple juice was 94% or higher for added levels of 25, 50, 100, 150 and 200 μg/l [23]. The percentage recovery of HMF was also found remarkably high for added levels of 1, 5 and 10 mg/l (Table 1).

4.2.1. Sensitivity

The sensitivity of the method was found very good for both HMF and patulin. The sensitivity achieved meets the requirements for quality control purposes taking into account the maximum permitted concentration levels of 50 μg/l for patulin and 5–10 mg/l for HMF well established in many countries and also recommended by the WHO and the IFFJP.

Detection sensitivity of patulin was approximately two times greater than HMF. On the basis of a 3:1 signal/noise ratio, the lower limits of detection for HMF and patulin were determined to be <0.01 mg/l and <5 μg/l, respectively. Most of the previous methods using spectrophotometry or liquid chromatography are not sensitive enough to measure relatively low amounts of HMF in fruit juices [1,7,16,24]. Kermasha et al. have reported an HPLC method which has a limit of detection of 0.025 mg/l HMF in apple juice [19].

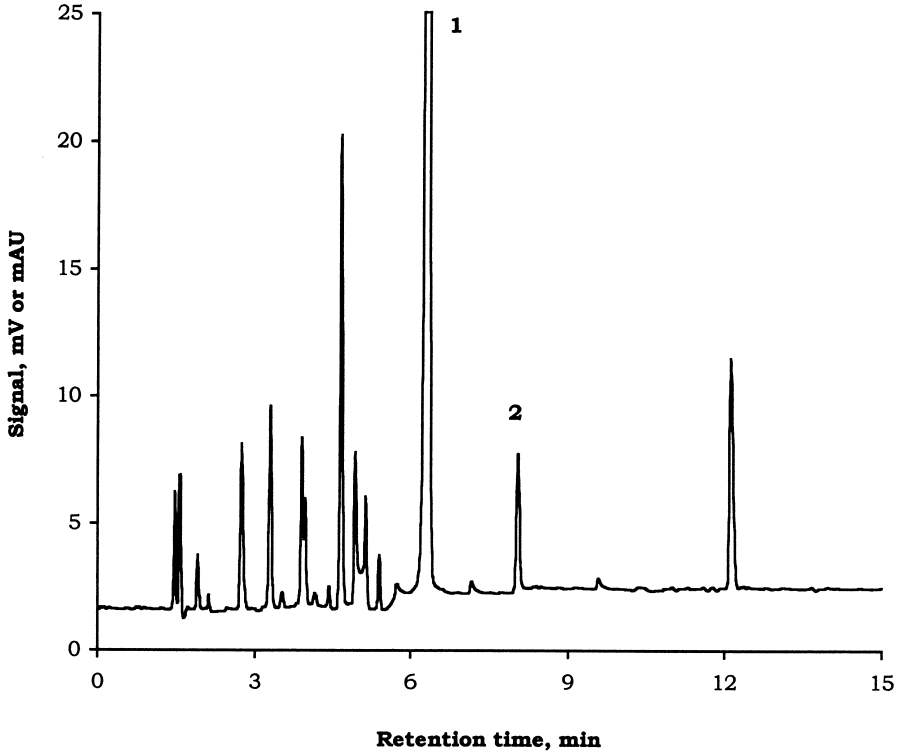

Fig. 3. Apple juice chromatogram, (1) HMF, (2) patulin (respective concentrations are 1.06 mg/l for HMF and 9.8 μg/l for patulin in apple).

4.3. Applicability

The applicability of this method was tested analysing large number of apple juice samples obtained from the manufacturers in Turkey. The results of HMF analyses were compared with the colorimetric toluidine–barbituric acid method. As low as 0.0054 mg/l of HMF could easily been detected in apple juice using the proposed reversed-phase liquid chromatographic method. However, HMF contents of apple juice samples lower than 0.25 mg/l could not been detected using the well-accepted colorimetric toluidine–barbituric acid method.

The present method was found useful for a large HMF concentration interval ranging from <0.05 mg/l to >10 mg/l in apple juices. The presence of relatively higher concentrations of HMF in the juice did not influence the measurement of patulin adversely. This point is considered as an advantage, because HMF content of juices tends to increase while patulin in juices is usually stable through an extended storage period.

The method was also found useful over a wide concentration range of patulin. As low as <5 μg/l and as high as >300 μg/l of patulin in apple juice could be detected easily without any noticed disadvantage.

Table 1
Recovery of HMF from apple juice[a]

Spikine level (mg/l)	Recovery (%)	RSD (%)	*n*
1.0	101.45	10.81	4
5.0	93.57	7.59	4
10.0	87.53	12.36	4

[a] Initial HMF concentration of apple juice (control, 0.0 mg/l spiking) was 0.008 ± 0.001 m/l ($n=4$).

5. Conclusion

In this study, simple, a precise and sensitive HPLC method which requires less chemicals and time for the determination of HMF and patulin is described. The method passed a series of validation tests including separation efficiency or resolution, sensitivity, reproducibility and applicability and found useful for routine quality control of apple juice. It is thought that the sensitivity achieved appears as an advantage particularly for the analysis of HMF in drinks in which HMF concentration is relatively low.

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