APPLICATION NOTE

UHPLC

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Analysis of the Mycotoxin Patulin in Apple Juice Using the Flexar FX-15 UHPLC-UV

Introduction

Patulin is produced by various molds, which primarily infect the moldy part of apples. Removing the moldy and damaged parts of the fruit may not eliminate all the patulin because some of it may migrate into sound parts of the flesh. Also, patulin can be produced within the fruit, even though it may not be visibly moldy. If moldy apples are used to produce apple juice, the patulin goes into the juice. It is not destroyed by heat treatments such as the pasteurization process. Patulin is a natural human toxin and therefore can have genetic affects within cells, including a developing fetus, the immune system and the nervous system. The recommended advisory level is 50 µg of patulin/kg in apple juice [50 parts per billion (ppb)].[1] Hydroxymethylfurfural (HMF), also 5-(Hydroxymethyl)furfural, is an organic compound derived from dehydration of sugars. HMF has been identified in a wide variety of heat-processed foods including milk, fruit juices, spirits, honey, etc.[2]

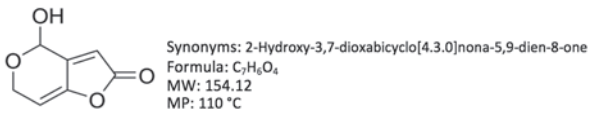

Figure 1. Structure and properties of patulin.

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This application note will demonstrate a rapid method for the identification and quantification of patulin in apple juice using high performance liquid chromatography with UV detection. In addition to method optimization and standard analysis, a number of apple juice samples for patulin were analyzed. The samples were randomly collected from the local market in Mumbai.

Patulin is a colorless to white crystalline solid. It is soluble in water, methanol, ethanol, acetone, and ethyl or amyl acetate and less soluble in diethyl ether and benzene.

Experimental

The PerkinElmer® Flexar™ FX-15, Ultra High Performance Liquid Chromatograph (UHPLC), equipped with a programmable wavelength UV/Vis detector was used for this application. Table 1 presents the detailed operating parameters of the UHPLC and the extraction process of patulin from juice. The instrument interaction, data analysis and reporting was completed with the PerkinElmer Chromera® data system.

Table 1. **Detailed instrument conditions used in the determination of patulin.**

Instrument	Flexar FX-15 High Performance Liquid Chromatograph
Analytical Column	Brownlee™ analytical DB AQ C18 1.9 µm x 100 x 2.1 mm column
Column Temp.	35 °C
Flow Rate	0.5 mL/min
Mobile Phase A	Water pH 4.0 with acetic acid
Mobile Phase B	Water : Acetonitrile (50:50)
Injection volume	25 µL
Wavelength	275 nm
Extraction Procedure	10 mL of apple juice + three extracts with ethyl acetate, combine the three extracts and add 4 gm $NaSO_4$. 25 mL of this extract was evaporated to dryness under a stream of nitrogen. The residue was dissolved in 300 µL of mobile phase A and injected in to the chromatographic system.

Stock Solution of Patulin: 200 µg/mL of patulin was prepared in ethyl acetate.

Stock Solution of HMF: 200 µg/mL of HMF was prepared in ethyl acetate.

Resolution Standard Solution: 100 µL each of the stock patulin and HMF were evaporated to dryness in a 10 mL flask, and the residue was dissolved in mobile phase A. This gave a 2 µg/mL mixture of patulin and HMF.

Preparation of Spike Solution: 250 µL of stock solution of patulin was evaporated to dryness under a stream of nitrogen and the residue was dissolved in mobile phase A. This gave a 5 µg/mL solution of patulin.

Calibration Curve: Varying volumes of 5 µg/mL patulin were spiked into 10 mL of juice samples to produce the following calibration curve (Table 2).

Table 2. **Scheme used for the creation of a six level calibration.**

Cal Level	Volume of Juice (mL)	Std Sol Added (µL)	Final Conc. Patulin (ppb)
1	10	20	10
2	10	40	20
3	10	80	40
4	10	100	50
5	10	160	80
6	10	200	100

Calibration: The UV detector was calibrated across the range of 10 to 100 ng/mL; each calibration point was run in duplicate to demonstrate the precision of the system. The average coefficient of determination for a line of linear regression was 0.998 for patulin. The calibration curve for patulin is depicted in Figure 3. The precision of the system across the calibration range is excellent. The chromatograms from the analysis of standard material are shown in Figure 4.

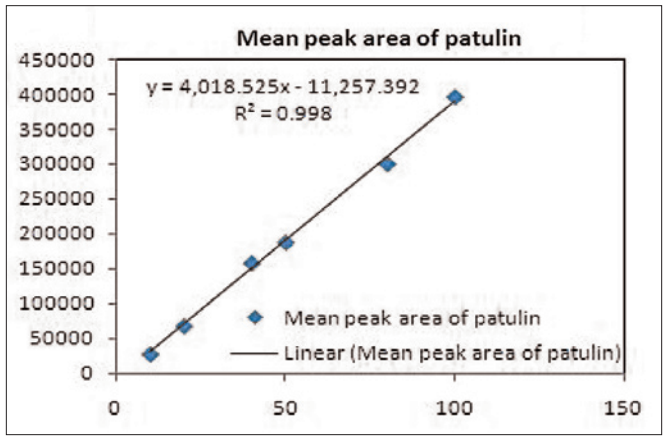

Figure 3. Calibration curve for patulin.

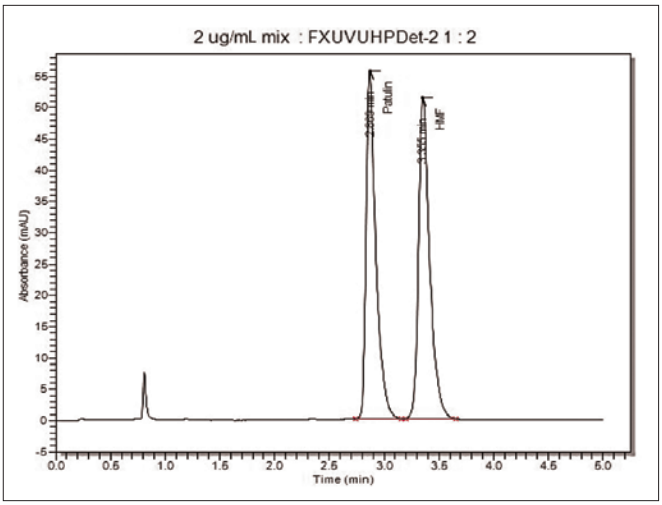

Figure 4. Example chromatogram for patulin.

The precision of the method was measured at both 5.0 and 10 ppb. The loss of precision below 10 ppb indicates the detection limit of this method to be approximately 5 ppb (Table 3).

Table 3. **RSD values for detection limit and quantification level.**

Sr. No.	Conc. of Patulin in ppb	Area of Patulin	Conc. of Patulin in ppb	Area of Patulin
1	5	14894.7	10	41189.2
2	5	18836.3	10	44147.4
3	5	25627.5	10	45305.8
4	5	33795.2	10	44107.4
5	5	30614	10	39631.3
6	5	30640.4	10	43282.5
Mean		25734.6		42943.9
S.D.		7455.87		2123.57
%RSD		**28.97**		**4.94**

Summary of Method Validation Experiment

Linearity: 10.0 ppb to 100 ppb of patulin

RSD for replicate analysis: for 10.0 ppb 4.94%

Detection level: 5.0 ppb

Quantification level: 10.0 ppb

Recovery study: at three levels for all the samples 80.52-109.48%

Sample Preparation: Samples were collected from the local Mumbai market. Samples included apple juice and apple squash. All the samples were refrigerated until analysis. Ten ml of juice sample was transferred into a 50 mL tube. The juice was extracted three times with 10 mL ethyl acetate. The three extracts were combined and 4 g of sodium sulphate was added to it, to remove moisture. 25 mL of the ethyl acetate layer was then evaporated (to dryness) under a stream of nitrogen. The residue was then reconstituted in 300 µL of mobile phase A, filtered through 0.2 µm nylon 66 syringe filter from Millipore® and 25 µL was injected in to the chromatographic system.

Method Validation

The recovery of the method was tested with the juice sample spiked at 3 different levels: 35, 50, 75 µg/L. The measured amount was 32.53, 47.49, 63.15 ug/L demonstrating that the technique is quantitative in its extraction of patulin from an aqueous matrix.

Results

Three samples of apple juice and one squash sample were analyzed using the method developed here.

Sample No.	Sample Details	Amount of Patulin Found in ppb
Sample 1	Juice T	N.D.
Sample 2	Juice S	N.D.
Sample 3	Juice R	N.D.
Sample 4	Apple squash	N.D.

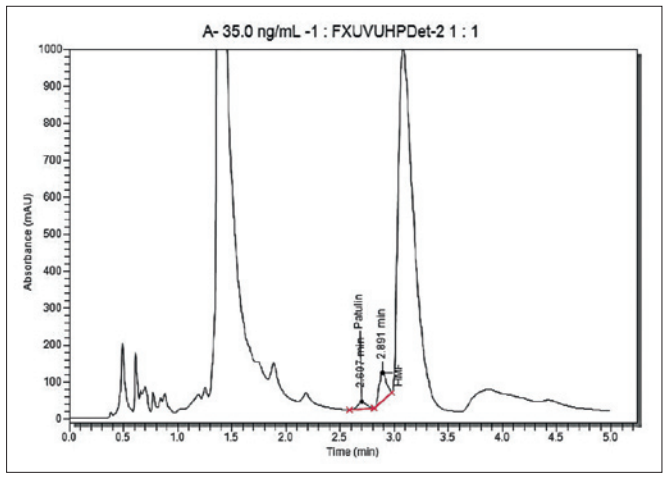

Figure 5. Chromatogram of patulin peak in sample.

Discussion and Conclusion

This application presents a method for the determination of patulin in apple juice. The method uses a Brownlee analytical DB AQ C18 1.9 µm x 100 x 2.1 mm column with a mobile phase of water of pH 4.0 with acetic acid. The flow rate is 0.5 mL/min. HMF elutes very close to patulin, therefore, it was necessary to demonstrate the separation between patulin and HMF. Ethyl acetate was used for the extraction of patulin and the extracts were treated with sodium sulphate to remove moisture. This was important because patulin may be destroyed when wet ethyl acetate is evaporated to dryness.[3] Also, a lower temperature of 40 ˚C was used for evaporation of the ethyl acetate layer, since patulin is not stable at high temperatures in ethyl acetate.

Alternately, a Brownlee validated AQ C18 100 x 2.1 mm x 3.0 µm can be used at a flow rate of 0.7 mL per min to achieve the same resolution between patulin and HMF.

The samples collected from the market were analyzed by the above method and the level of patulin determined. All the samples contained less than 5 µg/L of juice. The method was validated at several levels on juice matrix and the recovery values were between 80.52-109.48%.

References

1. Guidance on the control of patulin in directly pressed apple juice, http://www.newark-sherwooddc.gov.uk/ppimageupload.

2. Wikipedia.

3. AOAC Official Method 995.10, Patulin in Apple Juice, Liquid Chromatographic Method.

4. Journal of AOAC International, Vol. 90, No. 3, 879-883.

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