APPLICATION NOTE

Liquid Chromatography

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Analysis of Patulin in Apple Juice by UHPLC with UV Detection

Introduction

Patulin, its structure shown in Figure 1, is a toxic substance known as a mycotoxin produced by molds, such as *Penicillium*, *Aspergillus,* and *Byssochlamys*, which are frequently found on fruits, vegetables, cheese and grains. Patulin contamination is most common on damaged fruits, often intended for juices, pies and preserves, and is typically not found in alcoholic beverages or vinegar products due to it being inhibited during fermentation.[1] Its occurrence has been a significant issue in the processing of apple juices, as, due to its thermal stability, patulin decomposition is inhibited during pasteurization. Therefore, the proper handling and storage of fruits is very important in the prevention of patulin-producing molds.

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While not considered a particularly potent toxin, various studies have shown patulin to be genotoxic, which has led some to theorize that it may be a carcinogen. Based upon adverse effects of patulin in animal studies, the Food and Drug Administration (FDA) believes that, at some level of exposure, patulin may be a safety risk to humans.[1] In developing the action level for patulin, the FDA divided the population into three groups, focusing mainly on children, considering that small children can consume larger quantities of apple juice relative to their body weight. However, at this time, the FDA has not made any decisions on the level limits for individual groups, setting the general maximum limit to 50 µg/kg (50 ppb).[2]

Also basing its decisions on scientific data from animal studies, the European Union (EU), in Commission Regulation (EC) 1425/2003, has recommended that the maximum patulin intake limit be set to 50 µg/kg (50 ppb) for apple juice and apple products. In other beverages, such as spirit drinks and ciders, the recommended limit was also set to 50 µg/kg. For solid apple products, such as apple puree, a 25-µg/kg (25 ppb) limit was recommended. However, for any apple products intended for infants or young children, the limit was set to 10 µg/kg (10 ppb).[3]

Conventional methods for patulin testing have used multistep liquid-liquid extraction (LLE) or recently developed methods using SPE (solid-phase extraction). However, many of these methods require multi-step sample preparation and some use large quantities of solvents and chemicals.[4,5] Therefore, the focus in this work was to develop a simple, robust, and reliable LC method for the analysis of patulin in apple juice.

Method conditions and performance data, including linearity and repeatability, are presented.

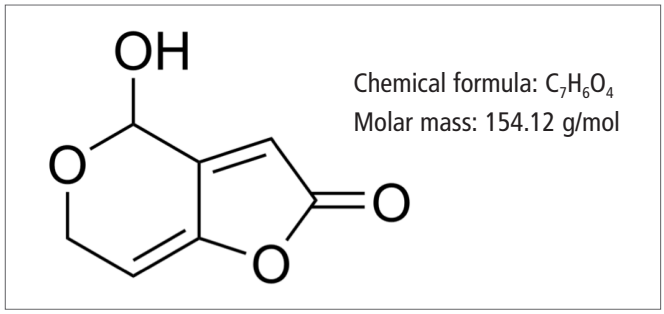

Figure 1. Chemical structure and properties of patulin.

Experimental

Hardware/Software

For the chromatographic separations, a PerkinElmer UHPLC system was used, configured with a solvent/sample management module, integrated vacuum degasser, column heater, and UV/Vis detector.

Method Parameters

The LC method parameters are shown in Table 1.

Table 1. LC Method Parameters.

Column:	PerkinElmer Brownlee™ HRes DB AQ C18 1.9 µm, 2.1 x 100-mm (Part# N9303919)
Mobile Phase:	Mobile Phase: 99:1 water/methanol; pre-mixed
Analysis Time:	5 min.
Flow Rate:	0.55 mL/min.
Pressure:	9900 psi/660 bar (maximum)
Oven Temp.:	35 °C
Detection:	275 nm
Injection Volume:	5 µL
Sampling (Data) Rate:	5 pts./sec

Note: It is recommended to wash the column for 30 minutes with a mixture of 50/50 acetonitrile/water after every 50 injections.

Solvents, Standards and Samples

All solvents, reagents and diluents used were either HPLC-grade or ACS-grade and filtered via 0.45-µm filters. Water was used as diluent, adjusted to pH 4.0 with glacial acetic acid.

The patulin standard was obtained from Sigma-Aldrich® Inc., Saint Louis, MO.

Experimental

Standard Preparation

A 200-µg/mL stock solution of patulin was prepared by adding 5 mg of the patulin standard to a 25-mL volumetric flask and filling to volume with ethyl acetate. A 4.8-µg/mL working standard was prepared by pipetting 0.6 mL of stock standard into a 25-mL volumetric flask and filling to volume with diluent. To maintain stability, both the stock solution and working standard were protected from light and refrigerated until further use.[4]

For retention time reproducibility determination a 0.3-µg/mL patulin standard was prepared by adding 75 µL of stock solution to a 50-mL volumetric flask and filling to mark with diluent.

For calibration of the working standard, the calibrants were produced via serial dilution of the working standard with diluent. The individual calibrant concentrations are provided in Table 2.

For calibration of the patulin in spiked apple juice, a 1.00 µg/mL patulin-spiked apple juice sample was prepared by adding 25 µL of the stock solution into a 5-mL volumetric flask and filling to volume. Thereupon, subsequent levels were prepared by serial diluting the 1.00 µg/mL-spiked sample. All calibrants were vortexed for 30 sec and filtered through a 0.45-µm nylon filter. For the spiked apple juice, individual calibrant concentrations are provided in Table 3. Each calibrant was injected in triplicate.

Table 2. Concentration of patulin at each calibration level.

Calibration Level	Conc. of Patulin (µg/mL)
1	0.0048
2	0.0096
3	0.0191
4	0.0382
5	0.0955
6	0.1910

Sample Preparations

Two apple juices, labeled Apple Juice A and Apple Juice B, were purchased at a local store.

An additional two samples, labeled Apple Juice C and Apple Juice D, were provided by a juice processing facility.

All juice samples were analyzed neat and refrigerated when not in use.

Results and Discussion

Using the method parameters described in Table 1, Figure 2 demonstrates the retention time of patulin at less than three minutes.

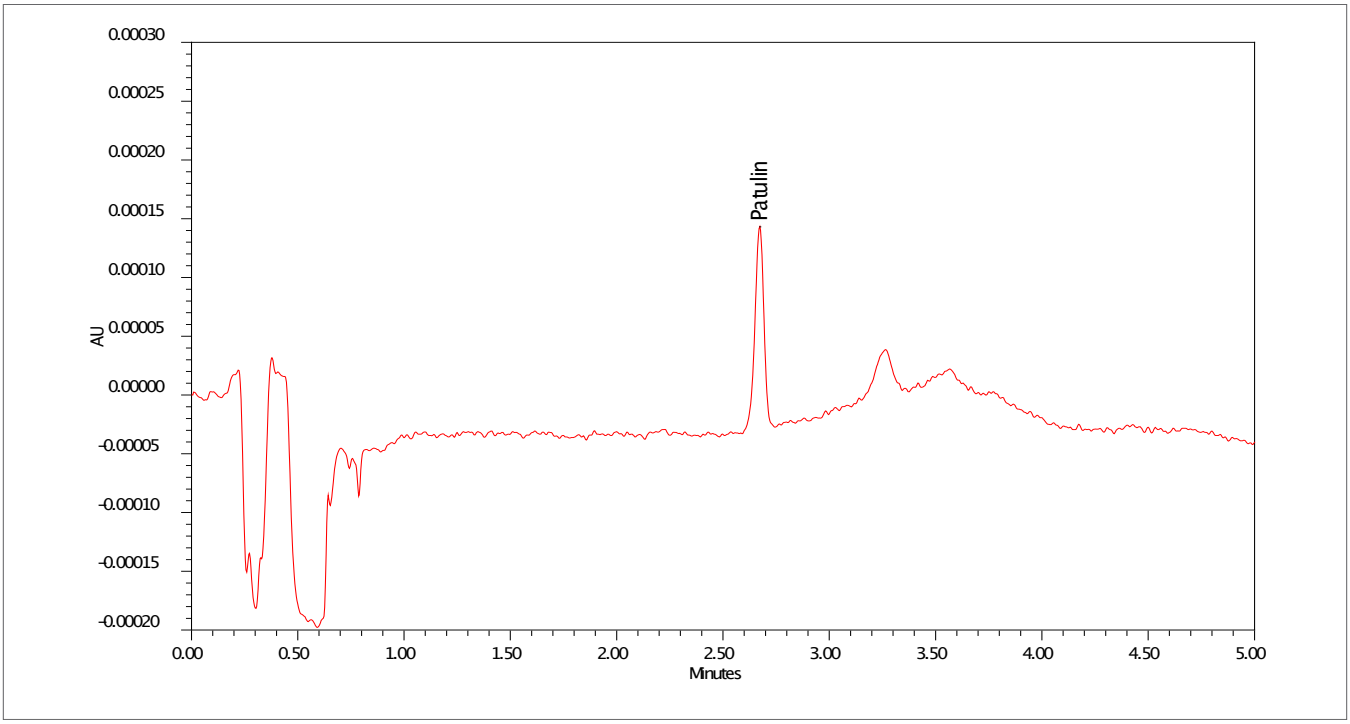

Figure 2. Chromatogram of the level-2 calibrant (0.0096 ug/mL) prepared in diluent; UV at 275 nm.

Figure 3 shows the overlay of 12 replicate injections of a 0.3 µg/mL standard, demonstrating excellent reproducibility. The retention time (RT) precision for patulin was 0.028% RSD.

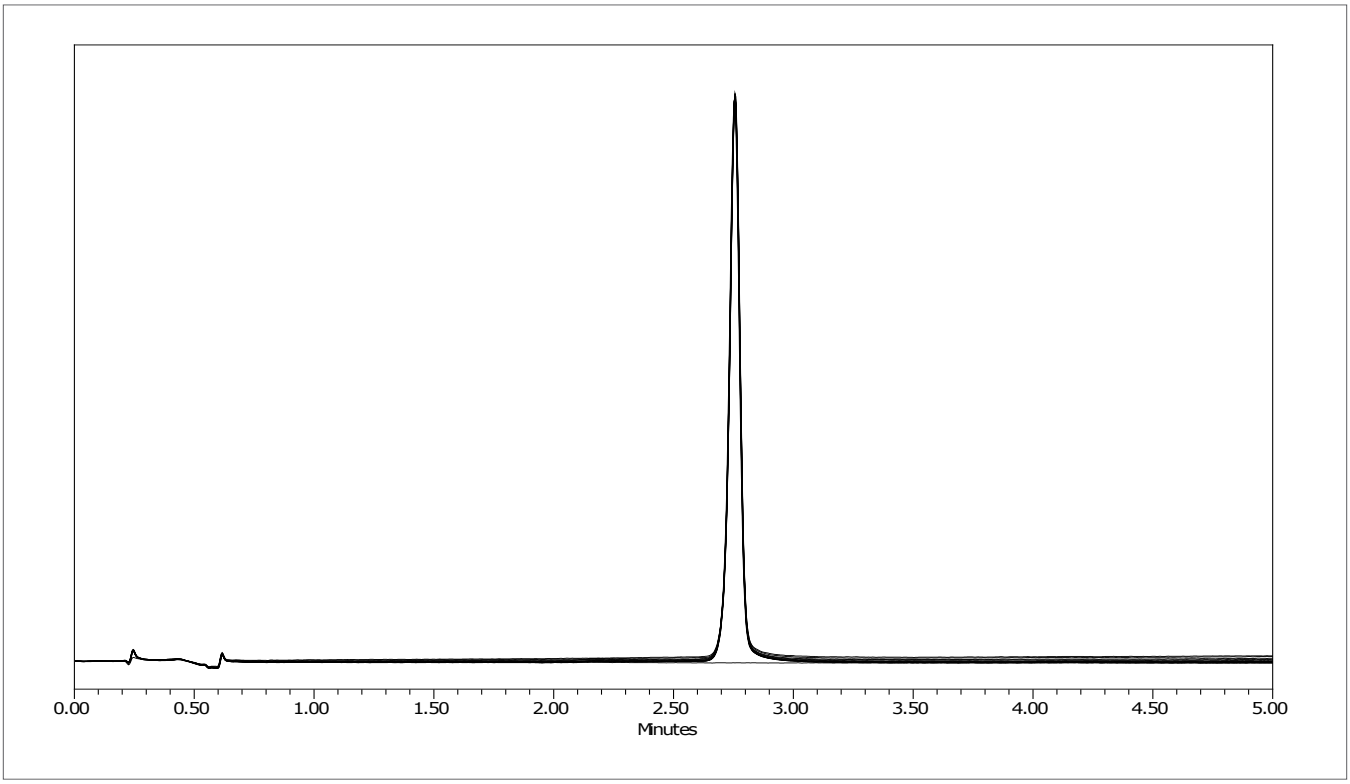

Figure 3. Overlay of 12 replicates of a 0.3 µg/mL standard; UV at 275 nm.

Figure 4 shows the calibration results for patulin in diluent, exhibiting an exceptional linear fit (R^2 = 0.99990; n=3). The two store-bought juices, Apple Juice A and Apple Juice B, were analyzed for the presence of patulin. As shown in Figure 5, no patulin was detected in either sample. To help target the analyte's expected elution time, each sample chromatogram was overlaid with the 0.3 µg/mL patulin standard.

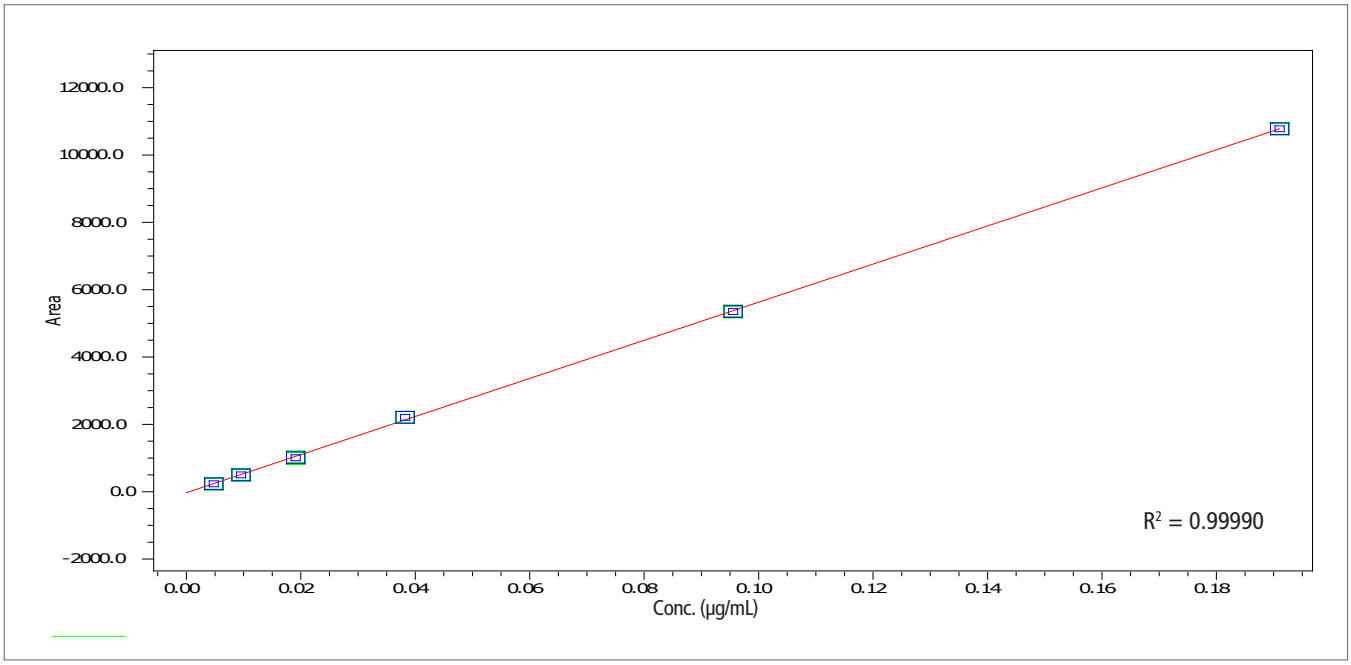

Figure 4. Results of 6-level calibration of patulin in diluent.

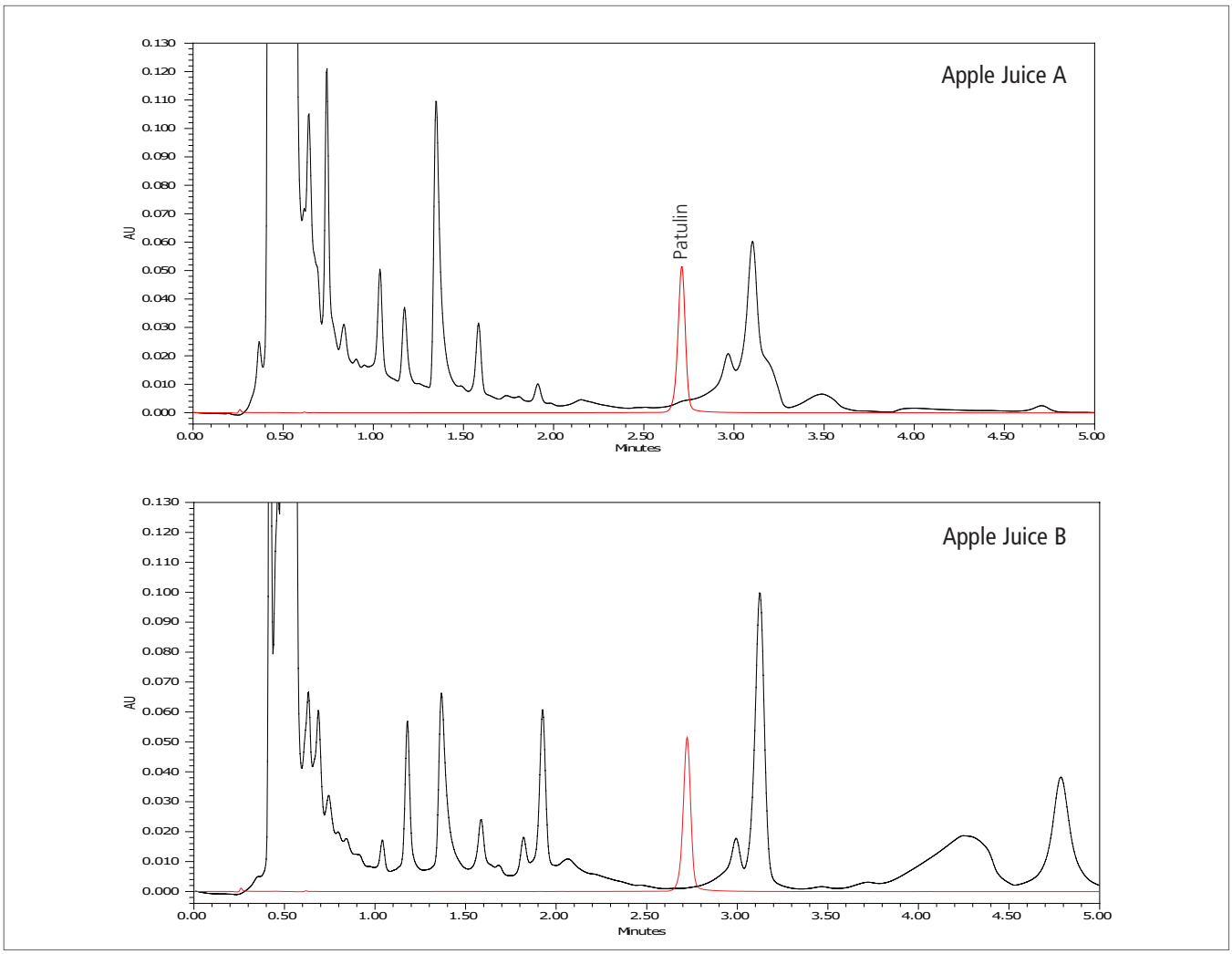

Figure 5. Chromatograms of Apple Juice A and Apple Juice B (black), and 0.3 µg/mL patulin standard in diluent (red).

To further establish the method's applicability in actual sample matrix containing patulin and to verify recoveries, a 5-level calibration set was created by spiking Apple Juice A with sequentially higher amounts of the 200-µg/mL stock solution, resulting in the concentrations shown in Table 3. Each calibrant was vortexed for 30 sec. and then filtered through a 0.45-µm nylon filter prior to injection. Once again, all calibrants were injected in triplicate.

Table 3. Concentrations of patulin in spiked Apple Juice A at each calibration level.

Calibration Level	Conc. of Patulin in Spiked Apple Juice A (µg/mL)
1	0.02
2	0.10
3	0.25
4	0.50
5	1.00

Figure 6 shows the Level 1 (0.02 µg/mL) spiked Apple Juice A overlaid with the 0.3 µg/mL patulin standard in diluent. The patulin is well resolved from other matrix peaks.

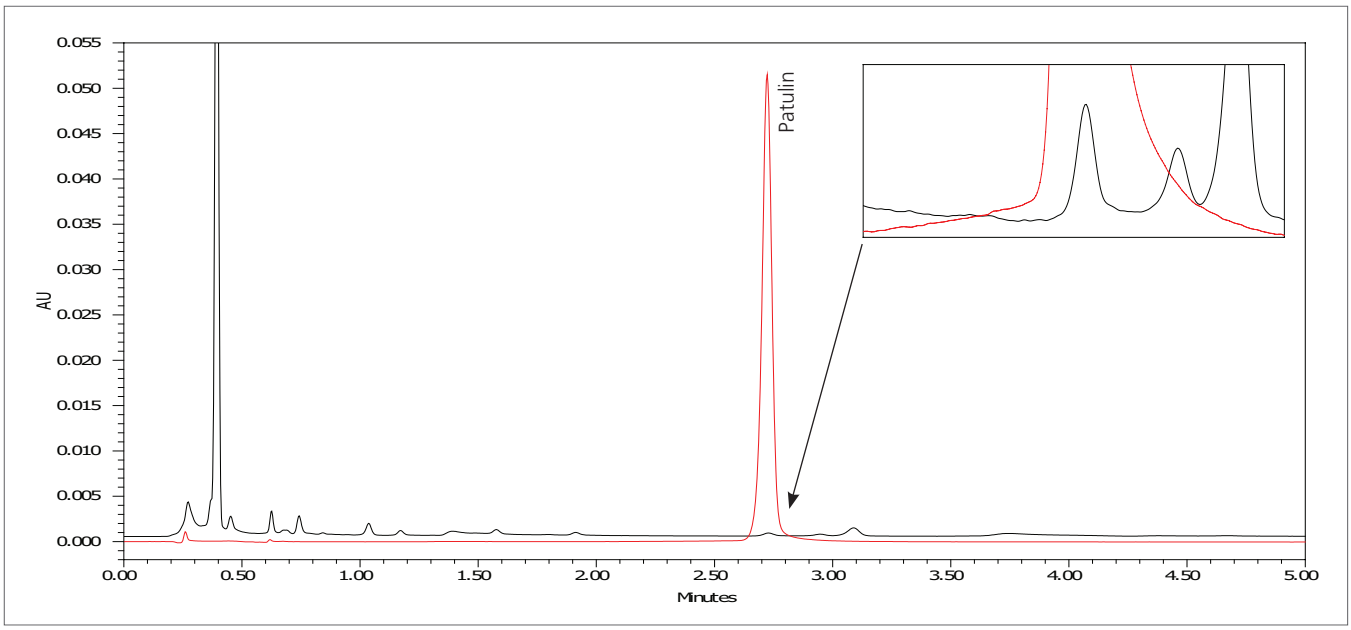

Figure 6. Chromatographic overlay of 0.02 µg/mL patulin-spiked Apple Juice A (black) and 0.3 µg/mL patulin standard in diluent (red).

Figure 7 shows the calibration results for the spiked patulin in Apple Juice A. These exhibited a good linear fit, with an R^2 value = 0.99932 (n = 3 at each level).

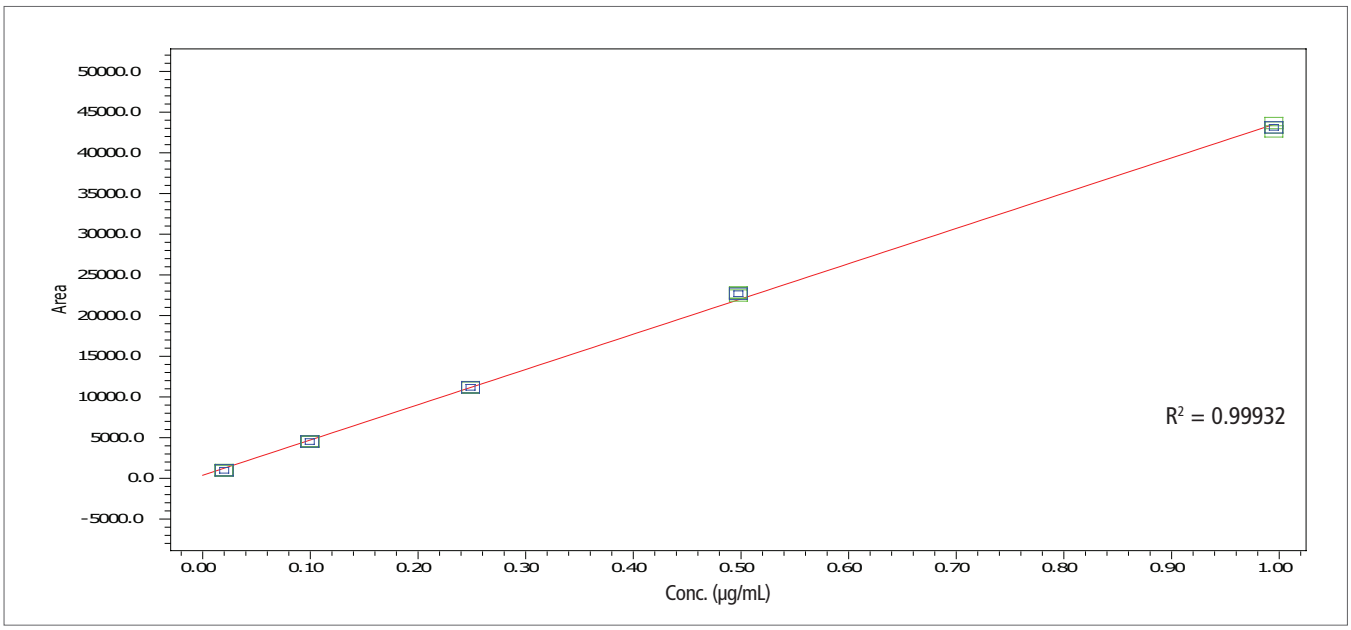

Figure 7. Results of 5-level calibration for patulin in spiked Apple Juice A.

For spiked patulin in Apple Juice A, the LOD and LOQ values were 0.0016 µg/mL (1.6 ppb) and 0.0053 µg/mL (5.3 ppb), respectively.

Based upon the initial calibration results for patulin in diluent, recoveries were calculated for each of the calibrant levels of spiked patulin in Apple Juice A (Table 4).

Table 4. Recovery of patulin in Apple Juice A at each spiked level, based on the initial calibration results for patulin in diluent.

Calibration Level	Conc. of Patulin (µg/mL)	Recovery of Patulin (%)
1	0.02	87.2
2	0.10	78.8
3	0.25	77.3
4	0.50	79.6
5	1.00	101.8

Apple Juice C and Apple Juice D, obtained from a juice manufacturing facility, were then analyzed. As shown in Figure 8, patulin was detected in both samples. Each sample was run in triplicate and the average patulin levels were calculated to be 0.071 µg/mL (71 ppb) and 0.706 µg/mL (706 ppb) for Apple Juice C and Apple Juice D, respectively. Both values were consistent with the provider's expectations.

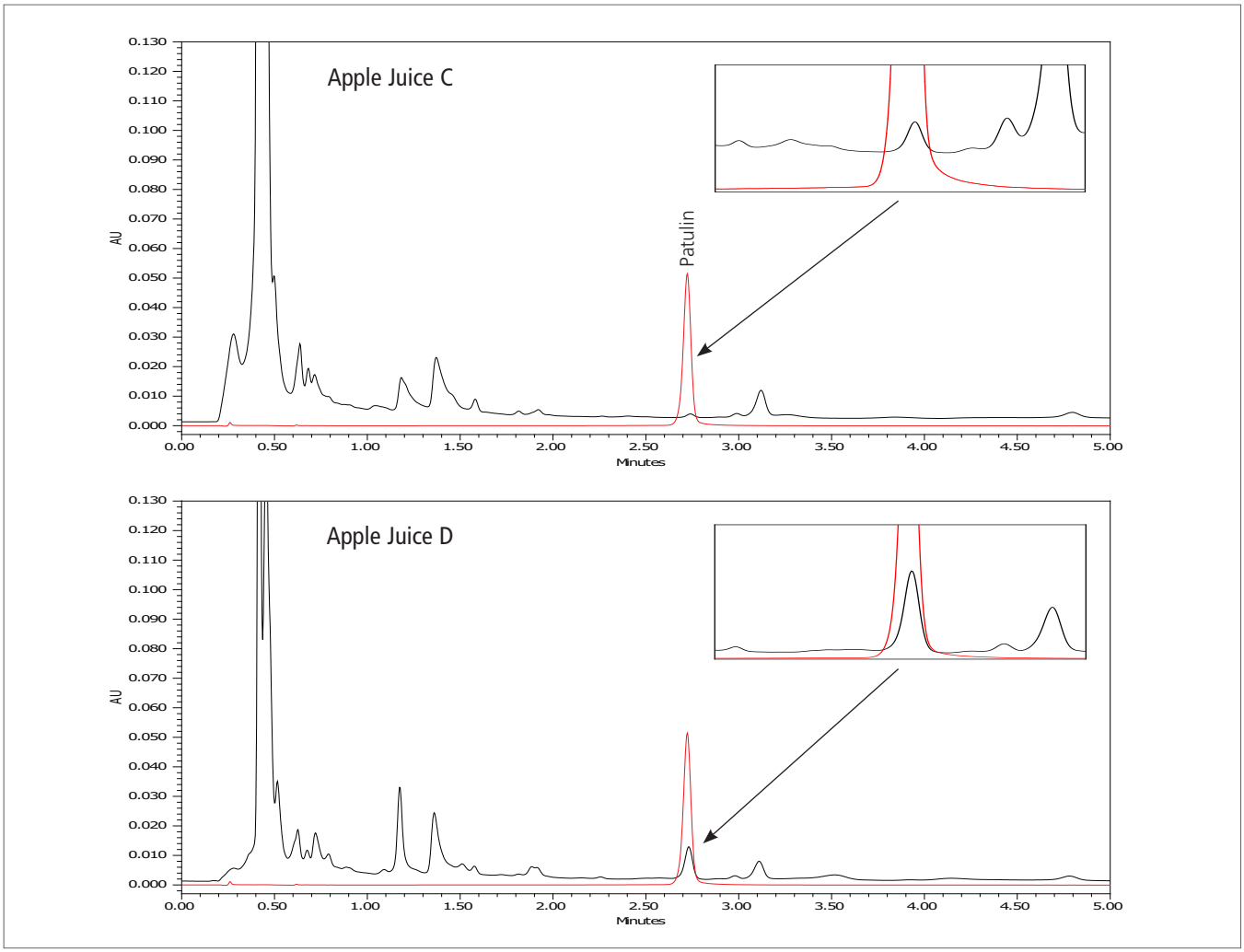

Figure 8. Chromatograms of Apple Juice C and Apple Juice D (black), and 0.3 µg/mL patulin standard in diluent (red).

Conclusion

The results obtained confirm the applicability of this method for the efficient, routine and robust chromatographic analysis of the patulin in apple juice. The analyte is well separated from other matrix components in under five minutes by UHPLC using UV detection. The results demonstrated excellent retention time repeatability, as well as very good linearity, over the tested concentration range. Thereupon, and considering both the 5.3-ppb LOQ level for patulin and the recovery results for actual samples, this application can be considered effective for monitoring patulin in apple juice and covers the EU's maximum allowable limit of 10 µg/kg (10 ppb) for small children. It is also well below the overall 50 µg/kg (50 ppb) limit currently recognized by the FDA.

References

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