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Note d'application

Rapid Analysis of Patulin Contamination in Apple Juice

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Abstract

To provide a solution for the early identification of contaminated batches of apple juice through the detection of unsafe levels of patulin in accordance with legislative guidelines.

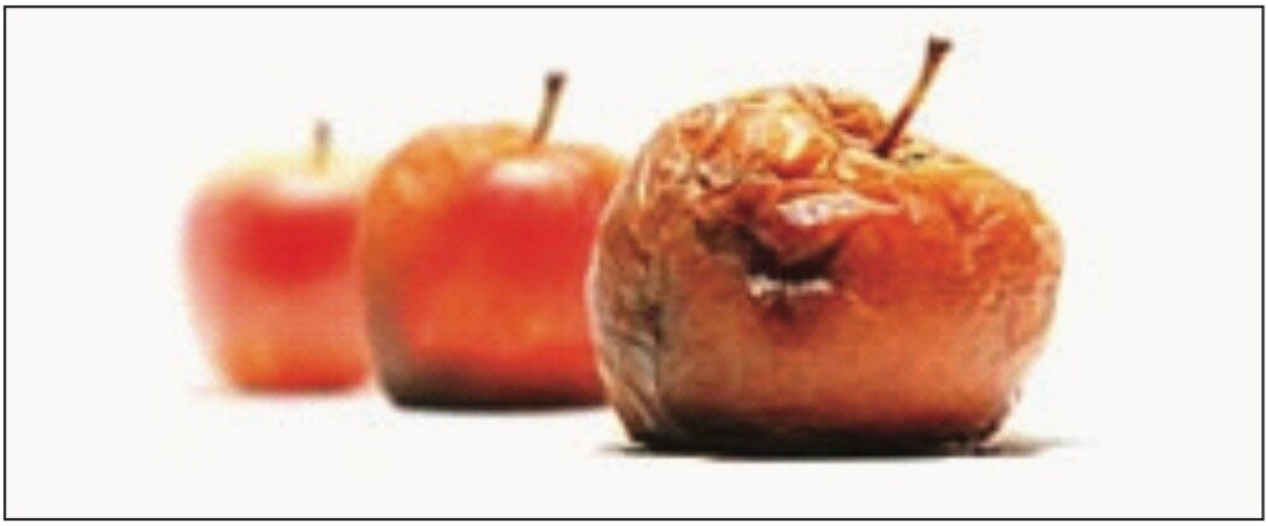
Benefits

- Increased efficiency while decreasing the need to retest samples resulting in increased productivity
- Cost savings will be made through lowering the use of lab consumables with the environmental impact of solvent usage also being reduced

Introduction

Patulin is a mycotoxin that is produced by several penicillium fungi commonly found on apples. Fallen fruit or apples that have been damaged by insects or birds, or bruised during handling, are more susceptible to the growth of patulin-producing molds.

The storage of apples under conditions that do not inhibit the growth of molds can lead to high levels of patulin. If these apples are then used to make juice, high levels of patulin may occur even when pasteurized, as thermal processing does not destroy this contaminant.



Exposure over time to high levels of patulin may pose a health hazard.[1] The U.S. FDA has established an action level for patulin in apple juice of 50 µg/kg as determined on single strength apple juice or reconstituted single strength apple juice.

In fact, one rotten apple (containing >10,000 parts per billion patulin) used along with 200 good apples would result in juice that could exceed the FDA's action level for patulin.[1]

Other legislation in Japan and the EU is also set at 50 µg/kg. Apple juice labelled for infants within the EU must comply with the lower level of 10 µg/kg. The toughest legislation currently is 5 µg/kg in Armenia.[2]

Achieving and going below legislative testing levels of patulin will lead to an increase in food safety and will ultimately lead to increased time and cost savings, as contaminated batches can be identified and destroyed early in the production process. This gives any juice producer the advantage over their competitors by ensuring a high quality product gets on to the market.

The following method describes patulin analysis using a solution which is able to exceed the requirements of current worldwide regulations.

Experimental

Extraction

The method is based on Waters Oasis HLB cartridges. This reversed phase cartridge contains a sorbent that is a co-polymer made from a balanced ratio of two monomers; the lipophilic divinybenzene and hydrophylic n-vinyl pyrrolidinone.[3]

Cartridge:	Waters Oasis HLB 3 cc /60 mg
Condition:	3 mL methanol 3 mL water
Load:	2.5 mL sample
Wash 1:	3 mL 1% $NaHCO_3$ (1g/100mL)
Wash 2:	1 mL 0.1% acetic acid Dry under vacuum
Elute:	2 x 1.5 mL 10% ethyl acetate in methyl t-butyl ether (MTBE)
Reconstitute:	500 µL water

Chromatographic Conditions

LC system:	ACQUITY UPLC System
Column:	ACQUITY UPLC BEH Shield RP_{18} 2.1 x 100 mm, 1.7 µm
Column temp.:	40 °C
Sample temp.:	4 °C

Flow rate:	600 µL/min
Mobile phase A:	Water + 0.1% NH_4OH
Mobile phase B:	Acetonitrile + 0.1% NH_4OH
Gradient:	0.00 min 99% A 1.80 min 99% A 2.30 min 10% A 2.80 min 10% A 2.81 min 99% A
Total run time:	4.5 min
Injection volume:	20 µL, full loop injection
Detection:	UV (PDA), 276 nm

The mobile phases both contain 0.1% ammonium hydroxide that at initial conditions gives pH of 10. Historically, silica columns would not tolerate such alkaline conditions as nucleophilic attack by hydroxide ions cause the silica to dissolve.

The nature of the Bridged Ethylene Hybrid (BEH) particles, uniquely found within the ACQUITY UPLC BEH Shield RP_{18}, allows high pH mobile phases to be used as six siloxane bonds would need to be broken to hydrolyse one BEH particle.[4] This gives the increased stability at high pH.

With an embedded carbamate polar group within the C_{18} ligand, this shield group enables the column to be compatible with 100% aqueous mobile phase composition[5], reducing the risk of column de-wetting at the initial conditions.

MS Conditions

MS system:	ACQUITY TQ Detector

Ionization mode:	ESI negative polarity
Capillary voltage:	2.5 kV
Desolvation gas:	Nitrogen, 800 L/Hr, 450 °C
Cone gas:	Nitrogen, 50 L/Hr
Source temp.:	120 °C
Acquisition:	Multiple Reaction Monitoring (MRM)
Collision gas:	Argon at 3.5 x 10^{-3} mBar

Acquisition and Processing Methods

This data was acquired using Waters MassLynx Software, version 4.1. Incorporated into MassLynx, the IntelliStartTM technology automates optimization of MS parameters for the sample and also monitors the health of the MS system, reducing the time for operator-intensive troubleshooting and upkeep.

This data was processed using the TargetLynx Application Manager. This quantification package is capable of automating quality control checks such as calculating ion ratios, flagging analytical results above/below thresholds set by the user, as well as many other features.

Results and Discussion

Patulin was successfully chromatographed and separated from all interferences in the apple juice matrix. 5-hydroxymethylfurfural (Figure 1) is the main interference in this analysis as it shares a UV chromophore with patulin at 276 nm. This compound is created when fructose or sucrose is heated, such as during pasturization6. For accurate quantifiable UV detection the two compounds must be chromatographically separated. A minimum of baseline resolution must be achieved for these two compounds when using a UV detector.

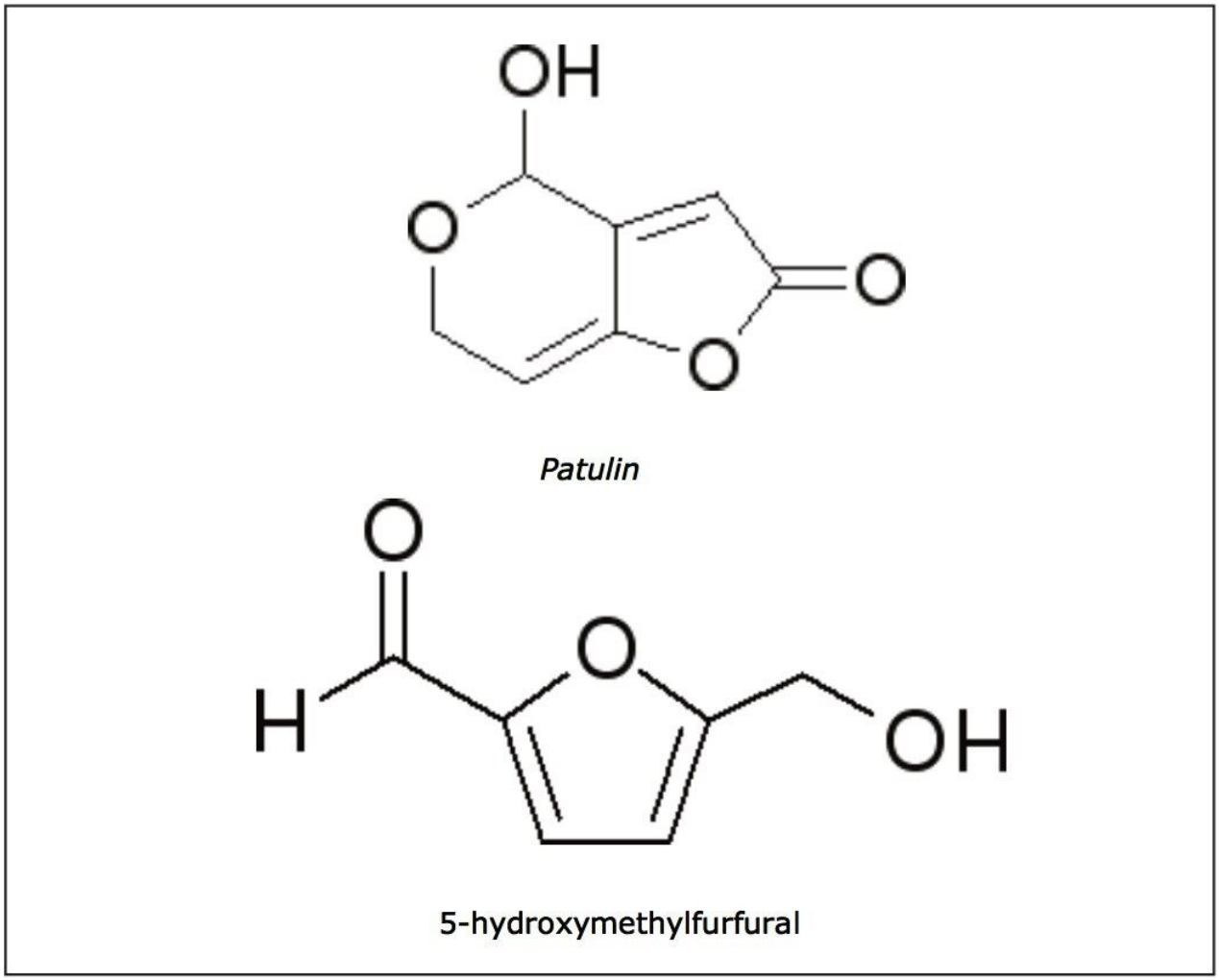


Figure 1. Patulin and 5-hydroxymethylfurfural.

Figure 2 shows the chromatograms from the UV (PDA). This rapid UPLC analysis gives a retention time of approximately 1.1 minute for patulin and 1.4 minutes for HMF without compromising resolution

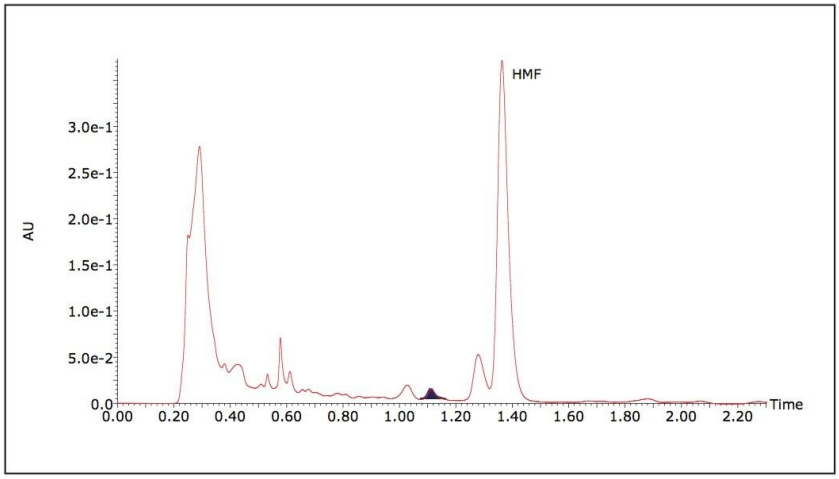


Figure 2. Apple juice extract at 50 µg/kg containing patulin and 5-hydroxymethylfurfural (HMF) at 276 nm.

Figure 3 shows the calibration curve produced from spiked extracts of apple juice. ACQUITY PDA (UV - photo diode array detector) is able to quantify between 25–5000 µg/kg. This approach will give confidence two times below limits of the 50 µg/kg legislations applied in Europe, and Japan.

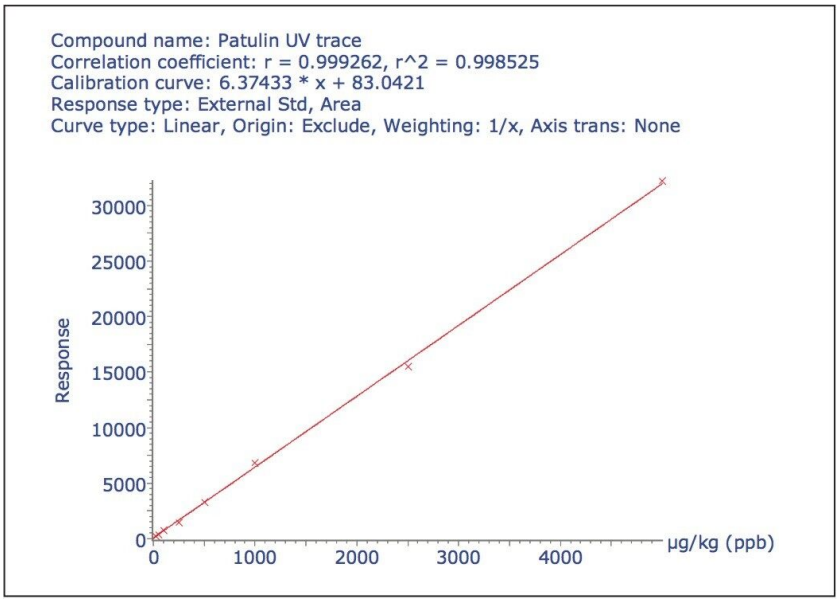


Figure 3. Apple juice extract calibration curve for patulin from 25 µg/kg to 5000 µg/kg using ACQUITY PDA at 276 nm.

By employing the selectivity of the ACQUITY TQD (tandem quadrupole mass spectrometer) in multiple

reaction monitoring mode, the levels of sensitivity for patulin analysis in apple juice is enhanced. Figure 4 shows the chromatography achieved at the legislative limit of 50 µg/kg with 16 data points across each peak.

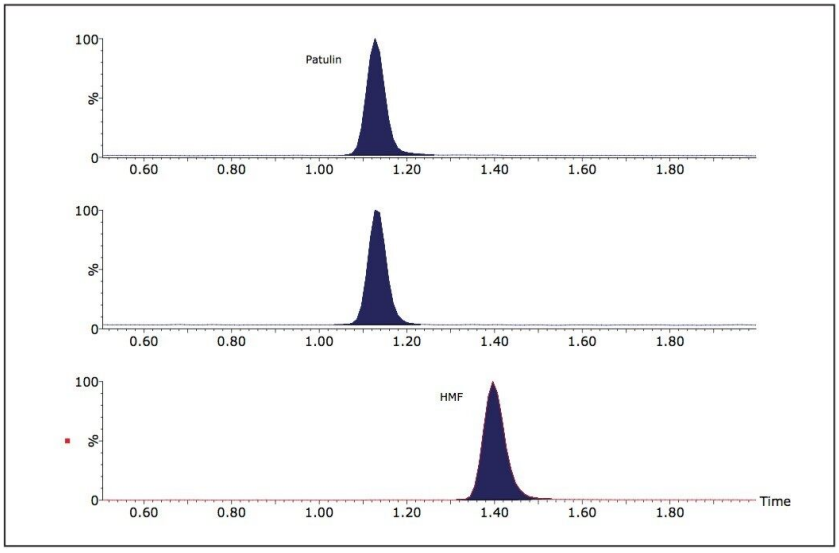


Figure 4. Apple juice extract at 50 µg/kg containing Patulin and 5-hydroxymethylfurfural in negative electrospray mode.

Figure 5 shows a calibration curve for apple juice extracts. The linear range is from 1–1000 µg/kg with the correlation coefficient (r^2) > 0.99. This gives the advantage of analyzing to levels of 10 times below European legislation for infants' apple juice and 50 times below the limit which applies to apple juice intended for adults in Europe, and Japan.

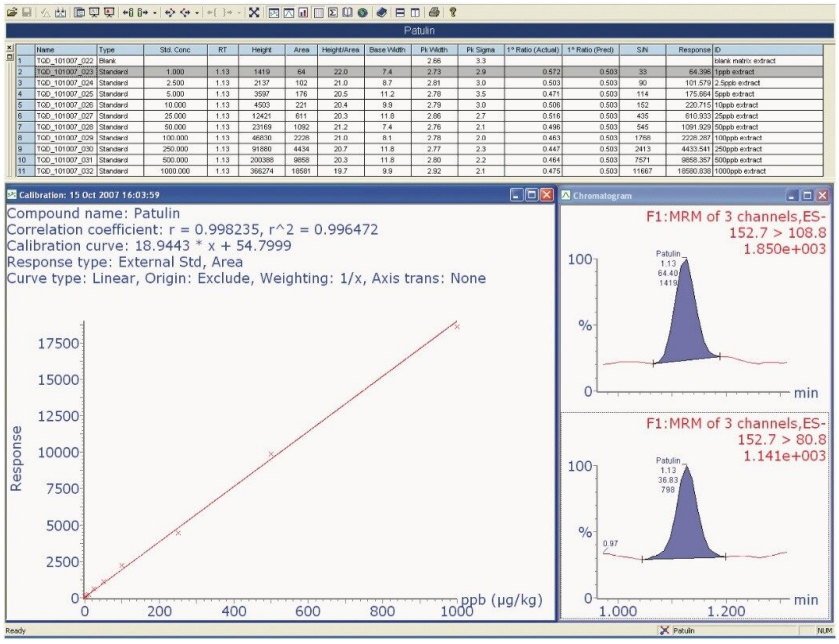


Figure 5. TargetLynx view showing linear range of analysis from 1–1000 µg/kg in apple juice matrix using ACQUITY TQD. The highlighted chromatogram is at 1 µg/kg.

The tandem MS technique also gives higher confidence in qualification due to higher specificity when compared to UV.

The Oasis extraction provides a sample clean up along with sample concentration which will lower the limit of detection for the assay. Oasis HLB does not suffer from the common historical problems suffered by many C_{18} cartridges with regard to drying out which often leads to sample retests[7], resulting in decreased productivity and increased costs. In some laboratories this can be as high as 10%.

Table 1 shows the percentage recovery (calculated using pre and post spiked apple juice) for this extraction. The average analyte recovery is approximately 90% using Oasis HLB.

Mean recovery (%RSD)	
5µg/kg	86.1% (13.6)
50µg/kg	95.4% (5.9)
500µg/kg	89.9% (17.5)

Table 1. Recovery data obtained from Oasis HLB extraction of patulin in apple juice. Four data points were measured at each level.

Ion ratio stability was also assessed and the average difference was found to be 3.4%. Figure 6 shows this plot with the mean value highlighted as a red line. All injections lie within a 20% tolerance limit as specified in EU legislation 2002/657/EC.[8] This legislation is for veterinary drugs but can be equally well applied to mycotoxin analysis.

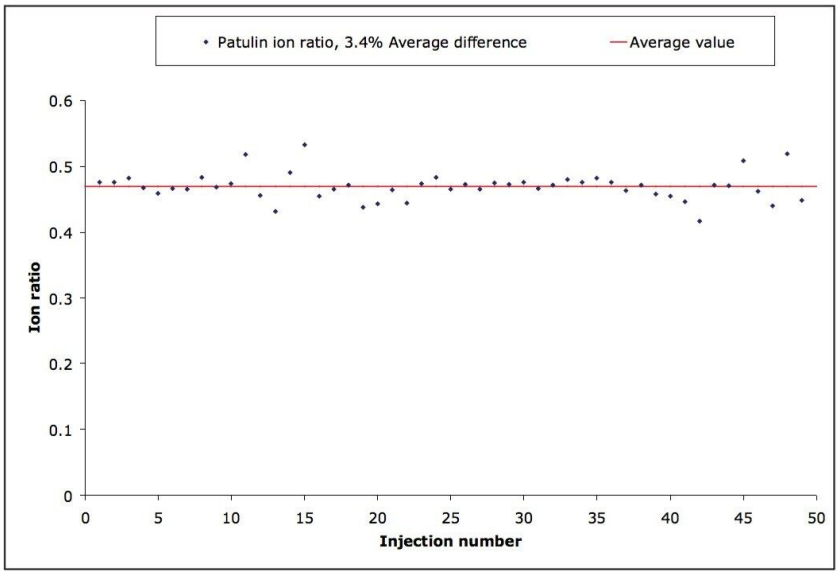


Figure 6. Plot showing changes in patulin ion ratio over a 50 injection sequence. The amount of compound injected onto the column from the sample extract is equivalent to 50 µg/kg in apple juice.

Conclusion

Patulin, which causes serious harm to human health, can be quantified in apple juice using the method described at levels below the EU, and Japanese legislation limits on ACQUITY PDA/TQD.

Lower levels of patulin quantification can be reached by using ACQUITY TQD giving more confidence in results close to or below legislative requirements.

The Oasis HLB extraction provides a sample clean up technique that demonstrates a high recovery while lowering limits of detection for this analysis. It can also lower the cost of sample analysis by reducing the number of repeat extractions.

Using the ACQUITY PDA, acceptable levels of quantitation can be achieved. However, the addition of MS/MS detection with the TQ Detector (Figure 7), will not only enable patulin analysis to be performed as described in this application note, but will also harness the benefits this powerful tool brings to the laboratory.

Tandem MS detection with ACQUITY TQD delivers enhanced signalto-noise and a dynamic range in apple juice enabling you to easily detect and quantify components of interest with speed and accuracy.

IntelliStart technology in this instrument is designed to reduce the burden of complicated operation, training new users, time-intensive troubleshooting, and upkeep. The small footprint of the ACQUITY TQD will give any laboratory an advantage as it removes the need for larger instrumentation.

The benefits of the Waters UPLC solution for a revenue conscious laboratory are shown with increased efficiency while decreasing the need to retest samples, resulting in increased productivity. Cost savings will be made through lowering the use of lab consumables with the environmental impact of solvent usage also being reduced.

Figure 7. The Waters ACQUITY UPLC solution featuring the PDA and TQ Detectors.

References

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2. Food and Agriculture Organisation of the United Nations website: http://www.fao.org/
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8. Commission Directive 2002/657/EC, *Off. J. of the European Communities* No. L221/8.

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