Use of cyclodextrin-based polymer for patulin analysis in apple juice

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Abstract

Penicillium expansum, one of the patulin producing fungi that causes decay on apple, is recognized as the main source of patulin contamination on apple and apple products. The widely used method for patulin analysis in apple juice is liquid-liquid extraction with ethyl acetate followed by HPLC-UV or LC-MS detection. Previous studies have shown cyclodextrin polymers to exhibit favorable adsorption properties for several classes of small organic molecules, including patulin in apple juice. In this study, an insoluble polymer composed of cyclodextrin crosslinked with 4,4'-methylenebis(phenyl isocyanate) was synthesized for use in the solid phase extraction of patulin from apple juice. Conditions investigated for this method were solvent for column conditioning, sample volume to load patulin on the column, solvent for washing, and solvent and volume for patulin elution and optimized recovery of patulin from the column. At the optimized conditions, the recovery and relative standard deviation (RSD) of patulin from apple juice spiked at 10, 20, 50, 80 and 100 ng mL⁻¹ were 78 and 20 %, 71 and 13 %, 78 and 17 %, 71 and 7.1 %, 67 and 2.9 %, respectively. Limit of quantitation (LOQ) of patulin in apple juice by this method was 10 ng mL⁻¹.

Introduction

Patulin (4-hydroxy-4*H*-furo (3, 2*c*)pyran, 2 (6*H*)-one) is a mycotoxin of apples and apple products. Toxicities of patulin were reported as gastrointestinal diseases¹⁾, genotoxicity²⁾ and DNA damage³⁾. The Codex Alimentarius Committee has recommended a patulin content less than 50 µg kg⁻¹ in apple products and in many countries, especially European countries set regulatory limits for patulin at 5–100 µg kg⁻¹⁴⁾.

Currently, liquid-liquid extraction with ethyl acetate followed by HPLC-UV⁵⁾ or LC-MS⁶⁾ is widely used as methods for patulin analysis in apple juice and other apple products. These methods are time-consuming and labor intensive. Immunoaffinity column based methods, which have been developed for detection of many mycotoxins in food and feed^{7–9)}, including aflatoxins, ochratoxin A and *Fusarium* mycotoxins. However, immunoaffinity methods are not reported for patulin yet, possibly due to the reactivity of patulin to thiol groups found in proteins, including antibodies. On the other hand, molecularly imprinted polymers

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(MIP) based clean up method was recently developed for patulin analysis¹⁰.

In this study, a cyclodextrin-polyurethane polymer consisting of β -cyclodextrin crosslinked with 4,4'-methylenebis (phenyl isocyanate) was used for solid phase extraction (SPE) of patulin in apple juice. Other cyclodextrin polymers have been developed and confirmed for adsorption to patulin, including patulin in apple juice¹¹. The cyclodextrin polymer used in this study was applied for patulin cleanup in apple juice as the solid phase in a method using SPE column cleanup followed by HPLC-UV analysis. Several important parameters were investigated to develop a more rapid and less labor intensive method for patulin determination. Conditions investigated for this method were solvent for column conditioning, sample volume to load on the column, solvent for washing, and solvent and volume for patulin elution from the column.

Materials and Methods

Chemicals and reagents Water was purified using a MILLIPORE system (AUTOPURE WT 100, Yamato, Scientific Co., Ltd, Tokyo, Japan). Stock standard solutions of patulin at $20.16 \mu\text{g mL}^{-1}$ was made from patulin in acetonitrile at $100.8 \mu\text{g mL}^{-1}$ (Biopure, Tulln, Austria) by acetonitrile. To make patulin-working solutions, an aliquot amount of patulin-stock standard solutions was put into a small amber glass vial and diluted with water acidified with acetic acid at pH 4.0. These solutions were stored at 4°C until used.

Acetonitrile, ethanol, sodium bicarbonate, acetic acid, hexane and diethyl ether were HPLC grade or GR grade and purchased from Kanto Chemical Co. Ltd (Tokyo, Japan). β -cyclodextrin, 4,4'-methylenebis (phenyl isocyanate), and anhydrous *N,N*-dimethylformamide (DMF) was purchased from Sigma-Aldrich (St. Louis, USA).

Cyclodextrin polymer for SPE column The polyurethane-cyclodextrin polymer with the cyclodextrin-crosslinker ratio of 1:10 was synthesized using the following procedure. Dried β -cyclodextrin (2 mmol, 2.27 g) was dissolved in 25 mL of anhydrous DMF in a 40 mL glass screw-capped vial using sonication (15 min, sealed vials). The solutions were purged with nitrogen for 5 minutes, and 4,4'-methylenebis(phenyl isocyanate) (20 mmol, 5.01 g) was added. The vial was sealed, vortexed, and then sonicated for 5 minutes. The vial was placed in a water bath (70°C) for 48 hours. Following polymerization, six vials of the cyclodextrin polymer were combined and the material was washed by vacuum filtration with excess acetone. The material was sonicated and washed with acetonitrile, water, and ethanol followed by a final rinse with excess water. The polymer was air dried by vacuum filtration, ground with a coffee grinder, and fractions between 38-75 microns were collected by sieving. Acetone sedimentation (3 x 200 mL) was employed to remove fine particles. Finally, the particles were sonicated in water (200 mL), vacuum filtered, and dried by vacuum filtration. 50 mg of the cyclodextrin polymer was packed in 3 mL plastic column (SUPELCO, Bellefonte, PA, USA).

Spiked sample with patulin preparation A clear type commercially available apple juice which contains $1.7 \mu\text{g kg}^{-1}$ patulin was used as apple juice sample. To make spiked samples with patulin, 2 mL of apple juice was taken into a 12 mm $\times$ 75 mm glass test tube and spiked with 40 μL of patulin standard working solutions, and mixed. Spiked samples with patulin at 5, 10, 20, 50, 80 and 100 ng mL^{-1} were prepared.

HPLC analysis Patulin was analyzed by LC-UV (Shimadzu LC- 10 series, Shimadzu Co. Ltd, Kyoto, Japan). HPLC system consisted of a LC-10AD pump, a SPD-10A UV-vis detector, a CTO-10ASvp column oven, a SIL-20AC auto sample injector, and a CBM-20A system controller. Patulin was separated by an ODS column (Synergi 4 μ Hydro-RP 80A, 4.6 mm i.d. x 250 mm) with a guard cartridge (Security Guard Cartridges AQ C18, 3.0 mm i.d. x 4.0 mm). The mobile phase was a mixture of water and acetonitrile (95 : 5, v/v). The flow rate was 1.0 mL min⁻¹. Temperature of the column oven was set at 40 °C. The detector was set at 276 nm. Twenty μ L of the extract were injected onto the column. The calibration curve was made with 0.14, 0.5, 1.0, 1.5, 2.0, 4.0 ng of patulin standard working solution. Limit of detection in patulin analysis was 0.04 ng (S/N = 3) and limit of quantitation was 0.14 ng (S/N = 10).

Method optimization

SPE column pre-condition. To prevent patulin breakthrough from SPE column during sample loading two different column pre-conditioning methods were investigated. One method was to condition the column with 5 mL of acetonitrile, water and ethanol¹¹⁾ and the other method was to condition the column with 5 mL of acetonitrile and water. Patulin levels at each stage of the SPE process were measured with HPLC.

Sample loading. From a previous study¹¹⁾, 1 mL of sample was loaded on SPE column for cleanup. To increase the scope of this investigation, three different spiked samples were evaluated to investigate the impact of sample loading on patulin retention. One sample was apple juice spiked with patulin at 50 ng mL⁻¹ (Sample A), the second sample was apple juice spiked with patulin at 10 ng mL⁻¹ (Sample B) and the third sample was prepared by diluting sample A five times with acidified water (Sample C). Sample A (1 mL) and 5 mL of Sample B and C were loaded on SPE column. Eluent of the first, second, third, fourth and fifth 1 ml fraction during sample loading and each step until final elution were collected. Patulin levels during sample loading and each step through final elution was measured with HPLC.

SPE column washing. The effect of wash volumes of sodium bicarbonate solution, acetic acid solution and hexane on patulin sample cleanup by SPE column was investigated. The amount of patulin breakthrough and recovery during sample loading and each step until final elution was measured with HPLC.

Patulin elution. Solvent and volume for patulin elution was optimized. Two different solvents for patulin elution from the column were examined, diethyl ether/acetonitrile (4:1)¹¹⁾ and acetonitrile. The first, second, third, fourth and fifth 1 ml fraction during elution was collected. Patulin levels during sample loading and each step until final elution was measured with HPLC.

Method validation. The following method was used for method validation. Prior to use, the SPE column was conditioned with 5 mL of acetonitrile followed by water (5 mL). The sample (1 mL) was loaded on the SPE column. The column was washed with 0.5 mL of an aqueous solution of sodium bicarbonate (1 %, w/v), an aqueous solution of acetic acid (1 %, w/v) and hexane. The column was dried by passing air with a pushing piston. Finally, patulin was eluted with 1.0 mL of acetonitrile at one drop per second, and this elution was repeated a second time. The solvent was dried under a gentle stream of nitrogen at 40 °C, and the residue was dissolved in 0.5 mL of acidified water, filtered over PTFE syringe filter (0.45 μ m, 13 mm), and collected into a 1.5 mL amber glass vial for further HPLC analysis.

This method were validated by examining patulin recovery, RSD and LOQ in spiked sample with patulin at 5, 10, 20, 50, 80 and 100 ng mL⁻¹. Each level of spiked samples with patulin at 5, 20, 80 and 100 ng mL⁻¹

were analyzed three times and samples at 10 and 50 ng mL⁻¹ were analyzed six times.

Results and Discussion

Patulin analysis can be simplified and carried out more quickly by avoiding liquid-liquid extraction step and loading apple juice onto the SPE column. Directly loading the apple juice sample onto the column was incorporated into the method optimization. To optimize the solid phase extraction clean-up of patulin from apple juice, we first investigated several parameters to condition, load, wash and elute patulin from the cyclodextrin polyurethane solid phase extraction column. The recoveries were analyzed by HPLC.

Method optimization

SPE column pre-condition. The breakthrough percentage and recovery of patulin from the SPE column for two different column pre-conditioning methods are shown in Table 1. The breakthrough percentage of patulin from SPE columns conditioned with acetonitrile and water was lower than conditioning with acetonitrile, water and ethanol. Recoveries of patulin into final eluent exhibited no differences. Therefore, we selected acetonitrile and water for the conditioning step in method development using the cyclodextrin polymer SPE column.

Sample loading. As shown in Table 2, three different samples containing the same overall amounts of patulin were loaded on the SPE column and recoveries from the SPE column are compared. Recoveries of patulin from SPE column were investigated using two loading on the SPE column methods (sample A and C). Recovery of patulin from Sample C loaded on the SPE column was c.a. 20 % lower than that from the SPE column loaded with the Sample A. These two samples contain the same amount of patulin and matrix from juice. These results show that charge volume influences patulin recovery. Also, patulin recovery of 5 mL of non-diluted apple juice (Sample B) was c.a. 20 % lower than that of diluted apple juice (Sample C). In these

Table 1. Effect of conditioning methods for patulin recovery

Elution Step	Patulin recovery (%) (n = 3)					
	Method A *			Method B **		
Sample loading	9.4	Tr.	N.D.	N.D.	N.D.	N.D.
Eluent of sodium bicarbonate solution wash	9.1	Tr.	N.D.	Tr.	Tr.	Tr.
Eluent of acetic acid solution wash	Tr.	Tr.	Tr.	N.D.	Tr.	N.D.
Eluent of hexane wash	N.D.	Tr.	N.D.	N.D.,	N.D.	N.D.
Elution (acetonitrile 1 mL x 2)	79	81	75	82	73	74
	(Ave.:78, RSD:3.7)			(Ave.:76, RSD:6.9)		

* Method A is conditioned with 5 mL of acetonitrile, water and ethanol.

** Method B is conditioned with 5 mL of acetonitrile and water.

N.D. = Not Detected

Tr = Trace

N.D.:less than LOD (0.04 ng)

Tr.:between LOD and LOQ (0.14 ng)

samples, the volume to load on the SPE column was same, but amount of matrix in sample was different. From these experiments, it is shown increasing the amount of matrix is associated with lower patulin recovery. From this study, the sample volume to load on the SPE column was set at 1 mL to maintain good recovery of patulin.

SPE column washing. The effect of column washing on sample recovery is shown in Table 3, increasing the volume of sodium bicarbonate solution, acetic acid solution and hexane for SPE column washing from 0.5 mL to 1.0 mL, lowers patulin recovery because breakthrough percentage of patulin from SPE column became higher. Also, there is no difference on the chromatograms of blank apple juice extract in two different methods. From these results, 0.5 mL was selected for the volume of SPE column washing solvent.

Table 2. Effect of sample loading methods for patulin recovery

Elution Step	Patulin recovery (%) (n = 1)		
	Sample A *	Sample B **	Sample C ***
Eluent of the first 1 mL fraction during sample loading	N.D.	N.D.	N.D.
Eluent of the second 1 mL fraction during sample loading		Tr.	N.D.
Eluent of the third 1 mL fraction during sample loading		Tr.	Tr.
Eluent of the fourth 1 mL fraction during sample loading		Tr.	Tr.
Eluent of the fifth 1 mL fraction during sample loading		8.6	7.8
Eluent of sodium bicarbonate solution wash	Tr.	Tr.	Tr.
Eluent of acetic acid solution wash	N.D.	Tr.	Tr.
Eluent of hexane wash	N.D.	Tr.	N.D.
Elution (acetonitrile 1 mL x 2)	74	33	58

* Sample A: Patulin spiked to 1 mL of apple juice at 50 ng mL⁻¹ (50 ng of patulin in a sample).

** Sample B: Patulin spiked to 5 mL of apple juice at 10 ng mL⁻¹ (50 ng of patulin in a sample).

*** Sample C: 5 mL of Sample A diluted 5-fold with acidified water (50 ng of patulin in a sample).

N.D.:less than LOD (0.04 ng)

Tr.:between LOD and LOQ (0.14 ng)

Table 3. Effect of volume of wash solvents for patulin recovery

Elution Step	Patulin recovery (%) (n = 1)	
	Method A *	Method B **
Sample loading	N.D.	N.D.
Eluent of sodium bicarbonate solution wash	Tr.	12
Eluent of acetic acid solution wash	N.D.	15
Eluent of hexane wash	N.D.	N.D.
Elution (acetonitrile 1 mL x 2)	82	64

* Method A: 0.5 mL of washing solvents for each step.

** Method B: 1.0 mL of washing solvents for each step.

N.D.:less than LOD (0.04 ng)

Tr.:between LOD and LOQ (0.14 ng)

Table 4. Patulin recovery and RSD in validation

Patulin concentration (ng mL ⁻¹)	Patulin recovery (%)	RSD (%)
5	Cannot be calculated (Tr., Tr., 170)	78*
10	78	20
20	71	13
50	78	17
80	71	7.1
100	67	2.9

* was calculated by peak area.

Tr. = Trace

Tr.:between LOD (0.04 ng) and LOQ (0.14 ng) and this recovery was lower than 60 %.

Each levels of spiked samples with patulin at 5, 20, 80 and 100 ng mL⁻¹ were analyzed for three times and spiked samples with patulin at 10 and 50 ng mL⁻¹ were analyzed six times.

Patulin elution. As shown in Fig. 1B, diethyl ether/acetonitrile (4:1) was used as solvent of patulin elution from SPE column, interference peaks exist near the elution time of patulin in chromatogram. However, when acetonitrile was used as solvent for patulin elution, interference peaks did not appeared (Fig. 1C).

Patulin recovery from SPE column at Eluent of the first, second, third, fourth and fifth 1 mL fraction during final elution were 75 %, Tr., N.D., N.D. and N.D., respectively. In addition, a chromatogram of the first 1 mL fraction of final eluent from SPE column from spiked sample with patulin at 50 ng mL⁻¹ was shown in Fig. 1D. From these results, 2 mL of acetonitrile was selected as patulin elution solvent.

Method validation. As shown in Table 4, at the optimized conditions, the recovery and RSD of patulin from apple juice at 10, 20, 50, 80 and 100 ng mL⁻¹ were 78 and 20 %, 71 and 13 %, 78 and 17 %, 71 and 7.1 %, 67 and 2.9 % respectively. However the recovery of patulin from apple juice at 5 ng mL⁻¹ varied from trace to 170 % (Av. less than 60%) and RSD was 78 %. The recovery of patulin from apple juice at 5 ng mL⁻¹ was not good and RSD was so large because the possibility of matrix effect on patulin analysis. Therefore, the LOQ for determination of patulin in apple juice by this method was 10 ng mL⁻¹.

Conclusion

The purpose of this study is to improve and simplify the HPLC-based analysis of patulin in apple juice. To achieve this, we develop and optimize a method based on a cyclodextrin polyurethane polymer as a novel sorbent in solid phase extraction clean-up. By this patulin analysis method, 10-100 ng mL⁻¹ of patulin in apple juice was quantitatively analyzed. This quantitative range covers with 50 µg kg⁻¹ of patulin in apple products as most countries set their regulatory limits.

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Fig. 1. HPLC chromatogram.

A) Patulin standard (1.0 ng).

B) Chromatogram of blank apple juice extract with diethyl ether/acetonitrile (4:1) as elution solvent of patulin from SPE column.

C) Chromatogram of blank apple juice extract with acetonitrile as elution solvent of patulin from SPE column.

D) Chromatogram of spiked sample with patulin at 50 ng mL^{-1} (acetonitrile as elution solvent).

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