Analysis of Patulin in Apple Juice by Diphasic Dialysis Extraction with in Situ Acylation and Mass Spectrometric Determination

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A procedure combining diphasic dialysis extraction with in situ acylation and gas chromatography/mass spectrometry (GC/MS) determination was developed for detection and quantification of the mycotoxin patulin in apple juice. Apple juice samples spiked with 4-*N*,*N*-dimethylaminopyridine were dialyzed using methane chloride and acetic anhydride inside dialysis tubing. Patulin was derivatized into its acetate and collected in the tubing after diphasic dialysis and was directly determined using GC/MS with the selective ion monitoring mode without further concentration and cleanup steps. Quantification was carried out by a calibration curve with an internal standard of correlation. The appropriate parameters of both dialysis and derivatization were examined. The linear range of the calibration curve was found to be 10−250 μg/L for patulin, and the limit of quantification was 10 μg/L. Levels of patulin ranging from 0 to 107.2 μg/L with 77−109% recovery were found in 10 apple samples. The technique combining diphasic dialysis extraction and acylation was demonstrated and showed potential for other applications.

Keywords: *Patulin; diphasic dialysis; in situ acylation; GC/MS*

INTRODUCTION

Patulin is a mycotoxin usually found in apples and apple products (Harrison, 1987) and is known to be produced by ∼60 species of molds belonging to >30 genera, mainly *Aspergillus* and *Penicillium* (Steiman et al., 1989). Patulin has been shown to be carcinogenic to many animals by subcutaneous injection (Harrison, 1987; McElroy and Weiss, 1993). Several countries regulate patulin at levels ranging from 30 to 50 μg/L (Van Egmond, 1989; Jelinek et al., 1989).

In the literature, the chromatography methods, including thin-layer chromatography (TLC) (Kubacki, 1986), liquid chromatography (LC) (Kubacki, 1986; Brause et al., 1996; Herry and Lemetayer, 1996), and gas chromatography (GC) (Ralls and Lane, 1977; Kubacki, 1986; Sheu and Shyu, 1995), have been applied for patulin analysis. The LC method, which was described by Brause et al. (1996), was recently validated by AOAC Method 995.10 (AOAC, 1998). However, the lowest detectable level of the AOAC method (20 μg/L) is just below the official limit, and this LC method lacks the more specific identification capabilities. Our laboratory reported a sensitive gas chromatography/mass spectrometry (GC/MS) method for patulin analysis using a direct acylation procedure before extraction to increase the stability and the recovery of patulin, as patulin acetate, and a following determination using GC/MS with the selective ion monitoring mode (SIM) gave a limit of quantification of 10 μg/L (Sheu and Shyu, 1995).

The major disadvantages of the LC and GC methods for patulin analysis are the extensive protocols for sample cleanup and concentration. A new technique called "diphasic dialysis" by Dominguez has been shown to be more convenient for the extraction of patulin from apple juice (Prieta et al., 1992; Dominguez et al., 1992; Prieta et al., 1993, 1994). Diphasic dialysis has usually been applied in the extraction of low molecular weight substances with low polarity from aqueous samples. In a classic diphasic dialysis apparatus, a semipermeable membrane separates two immiscible phases, such as water and organic solvent. Thus, the desired compounds pass through the membrane and move toward and are concentrated in the organic solvent phase during dialysis, whereas higher molecular weight or more polar substances are retained in the water phase by the membrane (Prieta et al., 1992; Sheu et al., 1996). This technique can provide a simple procedure for sample preparation with the advantages of labor and solvent savings (Dominguez et al., 1992; Sheu et al., 1996).

Dominguez's group extracted patulin from apple juice using diphasic dialysis with ethyl acetate as solvent and determined the patulin contents using TLC (Prieta et al., 1992; Dominguez et al., 1992) and HPLC (Prieta et al., 1993, 1994). However, the Prieta method still needed some complicated procedures such as concentration and cleanup steps between dialysis and determination and was also lacking in specific identification. If the acylation of patulin could be processed in situ during dialysis, patulin could be transformed into a more stable form as patulin acetate, and the recovery of extraction as well as the response of GC/MS detection could obviously be increased. In the present paper, a novel technique combining diphasic dialysis extraction and in situ derivatization together is presented. Different conditions and parameters influencing the recovery of patulin have been tested, and a method has been developed for the analysis of patulin in apple juice using this dialysis with in situ acylation technique and GC/MS confirmation of patulin acetate.

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Table 1. Recoveries of Patulin from Simulative Samples Containing 100 μg/L Patulin by Different Methods[a]

	method			
	A	B	C	D
acylation reagent spiked[b]				
outside the dialysis bag	Ac_2O	Ac_2O DMAP	DMAP	
inside the dialysis bag	DMAP		Ac_2O	Ac_2O DMAP
patulin recovery[c] (%)	28.7	17.1	78.8	12.3
RSD[d] (%)	17.6	26.1	12.7	22.4

[a] Each experiment was carried out in triplicate ($n = 3$). [b] Ac_2O, acetic anhydride; DMAP, 4-*N*,*N*-dimethylaminopyridine. [c] Recovery was calculated using the moles of patulin acetate yielded divided by the original moles of patulin. [d] Relative standard deviation.

MATERIALS AND METHODS

Samples. Applesauce, vinegar, and juice samples were obtained at a local market in Taipei, Taiwan.

Materials and Reagents. Patulin was synthesized from arabinose using a six-step procedure described by Seijas et al. (1989) and Bennett et al. (1991) in our laboratory. Methane chloride, acetic anhydride, 4-*N*,*N*-dimethylaminopyridine (DMAP), and preparative TLC (PTLC) plates (Merck Kieselgel 60 HF_{254}) were obtained from E. Merck (Schuchardt, Germany). Dialysis tubing (Spectra/Por 6 membrane MWCO 1000, 45 mm flat width, and Spectra/Por 4 membrane MWCO 12000−14000, 45 mm flat width) were obtained from Spectrum Medical Industries, Inc. (Los Angeles, CA).

Standard Preparation. *1. (4R,S)-4-Acetydroxyfuro[3,2-c]-pyran-2(4H,6H)-one (Patulin Acetate).* Patulin (500 mg, 3.25 mmol) and acetic anhydride (2 mL) were dissolved in dry methane chloride (20 mL), and then DMAP (1 g) was added. After stirring for 1 h at room temperature, the mixture was neutralized with saturated aqueous sodium bicarbonate. The lower organic phase was collected and evaporated under reduced pressure. The residue was partitioned again between saturated aqueous sodium bicarbonate (20 mL) and methane chloride (3 × 50 mL). The solvent of the combined organic layer was removed to give crude acetic patulin, which was then purified by PTLC (10:1 methane chloride/ethyl acetate, RF = 0.5) to give (*S*)-*O*-acetic patulin (457 mg, 72%) as an oily solid: MS(EI^+), *m*/*z* 196 (3), 154 (17), 137 (75), 136 (100), 110 (9), 81 (21), and 55 (58).

2. Stock Solutions at 200 μg/mL. Patulin (20 mg) was accurately weighed and dissolved in 100.0 mL of dry ethanol. The stock solution was stored in a freezer at 0−4 °C before use. The stock solutions of patulin acetate and nitrobenzene were prepared individually using the same method.

3. Patulin Simulative Sample at 100 μg/L. Five hundred microliters of a 200 μg/mL patulin stock solution was added into a 1 L volumetric flask and diluted to volume with deionized water. This stock simulative sample was stored in a refrigerator at 0−4 °C before use.

4. Patulin Working Solutions. From the patulin stock solution, patulin working solutions of 10, 50, 100, 150, and 250 μg/L were prepared by means of dilution with water. Each solution was spiked with the nitrobenzene stock solution as internal standard at a level of 50 μg/L.

Determination of Dialysis Parameters. Methane chloride (10.0 mL) as the extraction solvent was placed in previously hydrated dialysis tubing (MWCO 1 KDa), and the 100 μg/L patulin simulative sample (300.0 mL) was placed in a 500 mL flask. For the selection of the appropriate derivatization method, the reagents, including acetic anhydride (2.0 mL) and DMAP (100 mg), were added into the flask or the dialysis tubing according to the designed methods shown in Table 1. For the determination of the amount of acetic anhydride used for acylation, different volumes of acetic anhydride were added to the dialysis tubing, and DMAP (100 mg) was added into the flask. The dialysis tubing was enclosed tightly and introduced into the flask. After extraction by stirring for 24 h at 25 °C, the dialysis tubing was taken out. The upper aqueous phase was removed, and the lower organic phase was collected, dried, and measured in volume. Two microliters of the extract was injected into GC/MS.

A patulin acetate standard curve was previously made for the determination of conditions by injecting serially diluted patulin acetate standard solutions into GC/MS. The recoveries of patulin were calculated using the moles of patulin acetate detected divided by the moles of patulin in the original simulative sample. The method that yielded the highest recovery was selected for further experiments.

Diphasic Dialysis Extraction and in Situ Derivatization. The samples were previously spiked with 50 μg/L patulin to obtain spiked samples. For the analysis of the applesauce sample, 60 g of applesauce was diluted with 240 g of distilled water in prior to analysis. Methane chloride (10.0 mL) and acetic anhydride (1.0 mL) were placed in previously hydrated dialysis tubing, and the sample (300.0 mL), DMAP (100 mg), and nitrobenzene (50 μL of stock solution) as the internal standard were placed in a 500 mL flask. The dialysis tubing was enclosed tightly and introduced into the flask. After extraction by stirring for 24 h at 25 °C, the dialysis tubing was taken out. The lower organic phase was collected, dried, and measured in volume. The extract was placed into a 2 mL autosampling vial and injected into GC/MS in 2 μL.

GC/MS. Analyses were performed on a Hewlett-Packard (HP) 5890 Series II Plus gas chromatograph connected to an HP 7673 autosampler and an HP 5971 mass selective detector. An HP-5MS fused silica capillary column (30 m, 0.25 mm i.d., 0.25 μm film thickness, cross-linked to 5% phenyl methyl siloxane stationary phase) was used. The injector and detector temperatures were 200 and 250 °C, respectively. The column temperature was initially set at 50 °C for 2 min and then increased to 280 °C (ramp, 10 °C/min) and held for 3 min. The constant flow rate of helium was 1.0 mL/min (μ = 36.8 cm/s, measured at 60 °C). Mass spectra were obtained by means of electron ionization (EI) ranging from 50 to 300 amu. For patulin acetate determination, fragments at 196, 154, 137, 136, and 110 amu (*m*/*z*) were monitored using the SIM mode. Nitrobenzene was monitored at 77 and 93 amu (*m*/*z*).

Calculation. A calibration curve was made for the quantification of patulin. Each patulin working solution was extracted and derivatized using the same procedures used on the sample, and 2 μL of the extract was injected into GC/MS. The response of the peak area was measured at the retention times of nitrobenzene and patulin acetate. A linear regression plot of the calibration curve was constructed using the peak area ratio of patulin acetate to nitrobenzene versus the concentration ratio of patulin to nitrobenzene. The response factor of the calibration curve was calculated. The concentration of patulin ($C_{patulin}$, μg/L) in the original sample was calculated using

$$C_{patulin} = [(A_{patulinacetate}/A_{ISTD}) \times C_{ISTD}]/RF \qquad (1)$$

where $A_{patulinacetate}$ is the area response of patulin acetate; A_{ISTD} is the area response of the internal standard (nitrobenzene); C_{ISTD} = 50 μg/L, the concentration of the internal standard initially spiked to each patulin working solution and sample; and RF is the response factor of the calibration curve.

Three analyses of each sample were made, and each experiment was carried out in triplicate ($n = 3$).

RESULTS AND DISCUSSION

Dialysis and Derivatization Parameters. To facilitate the extraction procedure, the conditions of dialysis and acylation were investigated. Some parameters, such as the membranes, instruments, volumes of samples and solvent, and internal standard, were designed on the basis of previous studies (Dominguez et al., 1992; Prieta et al., 1993) and the experiences in our laboratory (Sheu and Shyu, 1995; Sheu et al., 1996).

Ethyl acetate was first used as the extraction solvent. After the usage of the methods listed in Table 1, all methods gave a poor solvent recovery (<30%) and

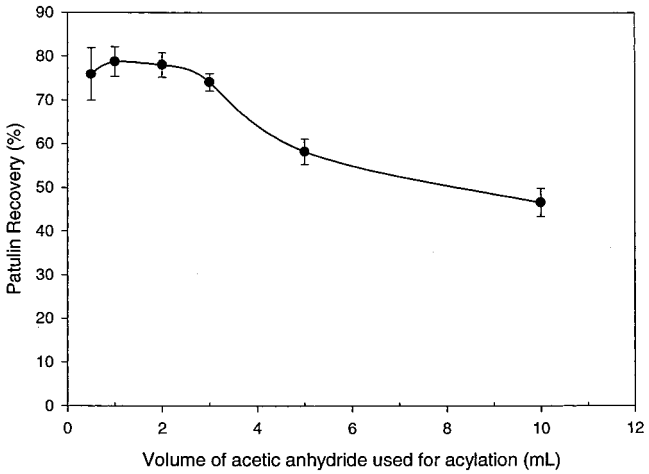

Figure 1. Recoveries of patulin by volume of acetic anhydride used for acylation. Three hundred milliliter simulative samples containing 100 μg/L patulin were extracted. Recovery was calculated using the moles of patulin acetate yielded divided by the original moles of patulin.

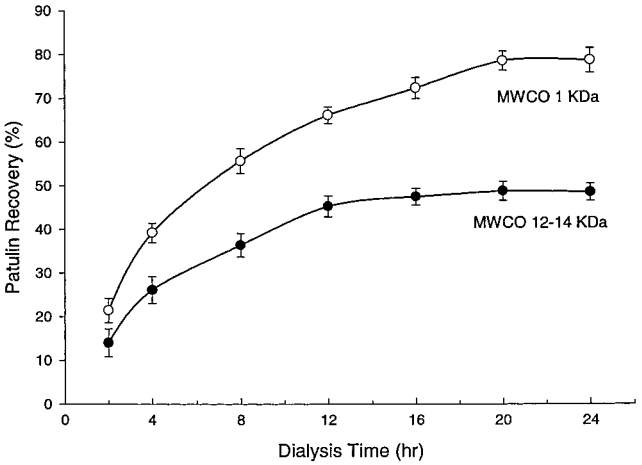

Figure 2. Recoveries of patulin extracted with MWCO 1K (○) and 12−14 kDa (●) membranes used at several time intervals. Simulative samples containing 100 μg/L patulin were extracted. Recovery was calculated using the moles of patulin acetate yielded divided by the original moles of patulin.

unquantitative results of patulin acetate. The low recovery of ethyl acetate inside the dialysis bag was believed to be caused by the existence of acetyl anhydride and hydrolyzed acetic acid, which increases the solubility of ethyl acetate in the aqueous phase. Methane chloride was then tried as a replacement for the ethyl acetate. Methane chloride was found to be more suitable for use as a dialysis solvent because higher solvent recoveries (>85%) were obtained for all methods in Table 1. The use of a derivatization activator (DMAP) was also found to be necessary for patulin acylation. No patulin acetate was found if DMAP was not included in this dialysis−derivatization system.

Four diphasic dialyses with in situ acylation methods were tested for the recovery of patulin acetate (Table 1). Method C with DMAP spiked to sample and acetic anhydride spiked to solvent yielded the highest recovery of patulin (78.8%, calculated as the moles of patulin acetate yielded). The lower patulin acetate recoveries of methods A and B might be due to the presence of acetic acid (hydrolyzed from acetic anhydride) in samples that decomposed acetic anhydride or reduced the solubility of patulin acetate in methane chloride. In method D, according to the coexistence of acetic anhydride and DMAP in the organic phase, acetic anhydride might be quickly hydrolyzed and DMAP might be quickly neutralized before the dialysis and acylation of patulin.

Acylation of patulin was presumed to mainly proceed in the organic phase due to the following two reasons. The first is that patulin, acetic anhydride, and DMAP are lipophilic. Acylation could probably take place more easily and more completely in an organic phase than in an aqueous phase. The second reason is the inferior recovery of patulin acetate found in method B, which provides only an aqueous acylation environment, relative to that found in method C. However, there was no direct evidence to prove these assumptions at the time.

The appropriate volume of acetic anhydride used for acylation was 1−2 mL (Figure 1) with the volume ratio of 10 mL of methane chloride to 300 mL of simulative sample. Figure 2 shows the recoveries of two membranes (MWCO 1 and 12−14 kDa) over time. The results show that 20 h was enough time for dialysis and acylation and that the membrane with MWCO 1 kDa yielded higher recovery (78.8%) than did the one with MWCO 12−14 kDa (48.8%). This could be explained by the lower solvent losses in a dialysis bag with a smaller MWCO, which provides higher overall recovery of solvent inside the bag. Those parameters that yielded higher patulin recoveries were applied in further experiments.

Table 2. LOQ of Patulin in Simulative Samples and Spiked Apple Juices by Means of Diphasic Dialysis Extraction and in Situ Acylation[a]

	patulin spiked (μg/L)	patulin found (μg/L)	recovery (%)	RSD[c] (%)
simulative samples	100.0	92.1	92	10.2
	50.0	45.2	90	12.8
	20.0	17.2	86	23.4
	10.0	8.1	81	18.3
	5.0	2.9	58	80.1
apple juice sample[b]	100	90.3	90	12.0
	50	45.4	90	16.5
	20	16.5	82	13.7
	10	7.9	79	20.2
	5	2.5	50	83.0

[a] Each experiment was carried out in triplicate ($n = 3$). [b] No detectable patulin found in this juice. [c] Relative standard deviation.

Calibration and Recovery. A calibration curve with an internal standard of correlation, made from simulative samples (working solutions), was made for patulin quantification. The five-point calibration curve was linear over the concentration range of 10−250 μg/L for patulin. Linear regression of the peak area ratio versus the concentration ratio gave a response factor of 0.0705 and a correlation coefficient of 0.9794. Although only a partial amount of patulin was extracted and derivatized, the rate of acylation, the extraction recovery, and the errors occurring in sample preparation could be calibrated. This curve was used to quantify simulative and apple samples.

For recovery determination, blank aqueous solutions and apple juice samples without patulin detected were spiked with different levels of patulin prior to extraction. Triplicate experiments ($n = 3$) of each sample gave the recovery results listed in Table 2. According to 5−100 μg/L patulin fortified samples, the mean recoveries ranged from 50 to 92%, and the relative standard deviation (RSD) ranged from 10 to 83%. The limit of detection (LOD), which was calculated as >10 times the signal-to-noise ratio (S/N), was 1 μg/L by a SIM mode detection. If the SCAN mode (scanning *m*/*z* 40−200

Table 3. Patulin Content of Some Apple Samples[a]

sample	apple product type	patulin found in blank (μg/L)	patulin found in spiked sample[b] (μg/L)	recovery (%)	RSD[c] (%)
A	juice	19.8	60.5	81	9.4
B	juice	107.2	161.7	109	8.2
C	juice	21.7	60.3	77	18.1
D	juice	46.0	92.9	94	17.0
E	juice	11.4	53.4	84	20.2
F	juice	9.8	49.5	79	45.3
G	vinegar	nd[d]	43.8	88	18.1
H	vinegar	14.2	53.3	78	17.0
I	sauce	nd	24.2	48	20.2
J	sauce	nd	27.9	56	45.3

[a] Each experiment was carried out in triplicate ($n = 3$). [b] Patulin of 50 μg/L was spiked. [c] Relative standard deviation. [d] Not detectable.

fragments) was applied for the detection, the LOD was only 20 μg/L. The detection by SCAN mode could obtain a complete mass spectrum but decreased the sensitivity of GC/MS. The limit of quantification (LOQ) was arbitrarily estimated to be 10 μg/L to keep a desirable recovery and an acceptable reproducibility (79 $\pm$ 20.2 %, as mean recovery $\pm$ RSD). This level (10 μg/L of patulin) was also within the linear range of the calibration curve.

Method Applicability. Patulin contents of 10 apple samples were determined using this dialysis−derivatization method (Table 3). In the six juice samples, the levels of patulin range from 9.8 to 107.2 μg/L with 77−109% recovery. Patulin was also found at a 14.2 μg/L level in a vinegar sample, but was not found in the other vinegar and two applesauce samples. In addition, the lower recoveries (48 and 56%) were observed in applesauce samples. This might be due to the higher viscosity and insoluble matrix content of the 1:5 diluted samples that reduced the diffusion and the dialysis of patulin.

The analytical characteristics of this method satisfy the patulin determination in apple juice. Recovery and reproducibility may be kept through a careful control of the dialysis conditions, for example, a constant stirring speed, a constant headspace in the dialysis bag, and a controlled temperature. Although the LOQ of this method (10 μg/L) is not as good as that of the Prieta method (1 μg/L) (Prieta et al., 1993, 1994), this method is a much simpler procedure for sample preparation in not having concentration and cleanup steps. These improvements are mainly due to the superior detection of GC/MS relative to HPLC−UV, which also provides a specific structural confirmation. A better LOQ could be obtained by a further concentration step after dialysis if more precise analysis is requested.

Compared to conventional methods using liquid−liquid extraction, this method provides two advantages: (1) Sample preparation is simplified. Each analysis took only 15 min of work by labor plus another 24 h for dialysis in our laboratory. (2) The amount of solvent used was decreased. Only 10 mL of solvent was used for each sample. The dialyzed extract could also be analyzed by HPLC or other instruments besides GC/MS.

In this study, an uncomplicated procedure using easily available semipermeable membranes and simple dialysis apparatus is employed for patulin quantification. This combined technique of diphasic dialysis extraction and the aqueous derivatization technique has been demonstrated to be workable. This technique shows the potential to be applied to the analyses of other compounds that must be derivatized before determination in an aqueous sample.

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