Note

A Quantification and Confirmation Method of Patulin in Apple Juice by GC/MS

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A sensitive and selective method for quantification and confirmation of patulin in apple juice by GC/MS was developed. By this method, patulin was precisely determined and confirmed down to the level of 1 and 5 $\mu\text{g/kg}$ in samples, respectively. Patulin was extracted with ethyl acetate from a sample and then hexane was added to the concentrated extract solution. Significant amounts of insoluble impurities were filtered off, followed by further clean-up by solid-phase extraction with combined silica gel and Florisil cartridges. The filtration step in a low-polarity condition was very effective to remove the impurities in the sample extract solution. The eluate from the cartridges was evaporated to dryness under reduced pressure and patulin was determined and confirmed by GC/MS after derivatization with 2.5% *N,O*-bis(trimethylsilyl)tri-fluoroacetamide ethyl acetate solution. Patulin was determined in the selected ion monitoring mode (m/z 226) and confirmed in the SCAN mode (m/z 40–340). The recovery from apple juice spiked with 10–500 $\mu\text{g/kg}$ ranged from 93.4 to 100%. The limits of detection and quantification were 0.1 ($S/N=3$) and 1 $\mu\text{g/kg}$ ($S/N=30$) of patulin in samples, respectively. Levels down to 5 $\mu\text{g/kg}$ of patulin in sample were readily confirmed.

Key words: patulin; apple juice; analysis; GC/MS; confirmation; mass spectrum

Introduction

Patulin is a mycotoxin produced by many species of *Penicillium*, *Aspergillus* and *Byssoschlamys*¹⁾. It is known that patulin is unstable under alkaline conditions ($\text{pH} > 7$). Patulin is toxic to many kinds of animals, such as mouse, rat, hamster, dog and chicken with LD_{50} values ranging from 5 to 170 mg/kg ²⁾.

Regulatory limits have been established for patulin by several countries in various foods, mainly apple juice. The most common limit is 50 $\mu\text{g/kg}$ ³⁾. In 2003 the Codex Committee on Food Additives and Contaminants adopted a maximum level of 50 $\mu\text{g/kg}$ for patulin. In Japan, a maximum level of 50 $\mu\text{g/kg}$ was set for patulin in apple juice by the Ministry of Health, Labor and Welfare on November 26 in 2003 and will come into force on June 1 in 2004⁴⁾.

Generally, analytical methods should be able to determine the object at the level of one tenth of the regulation limit. Furthermore, as patulin is a mycotoxin, a very toxic substance, we should confirm patulin in all positive samples. Therefore, a method, which can determine and confirm patulin at the level of 5 $\mu\text{g/kg}$ in sample, is needed. Among the methods so far developed for the analysis of patulin in apple juice, the method consisting of extraction with ethyl acetate, clean-up with sodium carbonate solution, concentration and

quantification by reversed-phase liquid chromatography (LC) with ultraviolet (UV) detection is most widely used and has been adopted as the AOAC official method⁵⁾. Although this method is applicable to determination of 20–200 μg patulin/L of apple juice⁵⁾, many interfering peaks on the liquid chromatogram often make it difficult to determine the content of patulin precisely. Further, it is also difficult to confirm patulin by UV spectroscopy, because the UV spectra of the interfering peaks near to patulin are very similar to that of patulin.

For confirmation of a given compound, comparison of mass spectra obtained by GC/MS is considered to be very reliable, because characteristic fragment ions are formed by electron impact ionization. LC/MS is regarded as inferior to GC/MS for confirmation, because only a few fragment ions are available under usual conditions and the fragment patterns of mass spectra vary with the conditions of HPLC and MS.

Although some methods using GC/MS have been developed for analysis of patulin in apple juice^{6–9)}, they are not sensitive enough to quantify and confirm patulin at the 5 $\mu\text{g/kg}$ level. Rychlik and Schieberle reported a highly sensitive method by GC/high-resolution mass spectrometry (HRMS) using ¹³C-labeled patulin as a surrogate¹⁰⁾. Although HRMS may be a highly sensitive and selective method, it has not been commonly

used because it is very expensive. Rupp and Turnipseed reported a confirmation method for patulin in apple juice by GC/MS⁶⁾. The extraction and clean-up procedures in this method were almost the same as those in the AOAC method. By this method, patulin in samples was confirmed at the level of 30–400 $\mu\text{g/L}$.

In this study, we developed a GC/MS method, by which patulin can be precisely determined with a quantification limit of 1 $\mu\text{g/kg}$ and confirmed down to the level of 5 $\mu\text{g/kg}$, one-tenth of the regulatory limit in Japan, in samples.

Materials and Methods

Reagents

Standard stock solution (100 $\mu\text{g/mL}$): 5 mg of patulin (Sigma Chemicals Co., St. Louis, MO, USA) was dissolved in 50 mL of ethyl acetate. This solution was stored in a refrigerator ($<5^{\circ}\text{C}$).

Standard working solution: The stock solution was diluted with ethyl acetate to make a concentration of 2 $\mu\text{g/mL}$.

Organic solvents: ethyl acetate and hexane of special grade and acetonitrile of HPLC grade were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Water for HPLC mobile phase: water purified with a Milli-Q purification system (Waters Co., Milford, MA, USA).

Cartridge columns: Sep-Pak Plus silica and Sep-Pak Plus Florisil were from Waters Co (Milford, MA, USA). A Sep-Pak Plus Florisil cartridge column was connected under a Sep-Pak Plus silica cartridge column. They were conditioned with 10 mL each of ethyl acetate and hexane before use.

Derivatizing agent: *N,O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was from GL Sciences (Tokyo, Japan).

Acetic acid solution (pH 4): Water was adjusted to pH 4 with acetic acid.

Filter: Polytetrafluoroethylene (PTFE) membrane filter combined with a pre-filter of glass fiber, 0.45 μm in pore size and 25 mm in diameter, was from Whatman Co. (Maidstone, Kent, UK)

Apparatus

GC/MS: GC-17A gas chromatograph with a QP-5000 mass spectrometer and a Class 5000 system as a data processor (Shimadzu, Kyoto, Japan).

HPLC system: Gulliver series (JASCO Co., Tokyo, Japan) integrated with a model PU-2089 pumping system, a model AS-1555-10 auto sampler injector, a model UV-2070 UV-VIS detector, and a Borwin system as a data processor.

Determination of patulin by AOAC official methods of analysis 995.10⁵⁾

A 5 mL aliquot of sample was extracted twice with 10 mL of ethyl acetate and the combined extract solution was washed with 2 mL of 1.5% sodium carbonate solution. The aqueous layer was extracted with 4 mL of

ethyl acetate. The ethyl acetate layers were combined and evaporated to dryness, dissolved in 1 mL of acetic acid solution (pH 4) and applied to an HPLC system.

Sample preparation of the proposed method

1. Extraction

After addition of 10 g of sodium chloride, 50 g of apple juice was extracted twice with 100 mL of ethyl acetate in a 300 mL separatory funnel by shaking for 10 min. Ethyl acetate fractions were combined and evaporated to dryness under reduced pressure at 35°C and then the residue was mixed well with 5 mL of ethyl acetate, and sonicated for 30 sec. This solution is hereinafter called "sample extract".

If the sample was an apple juice concentrate, 50 mL of water was added to 50 g of sample before extraction to reduce the viscosity. Then 20 g of sodium chloride was added and the mixture was extracted twice with 200 mL of ethyl acetate in a 500 mL separatory funnel by shaking for 10 min. The following procedure was the same as described above.

2. Clean-up and derivatization

Two mL of "sample extract" was pipetted into a 20–30 mL flask or tube, to which 18 mL of hexane was added and mixed well. Insoluble impurities were filtered off with a PTFE membrane filter combined with a pre-filter of glass fiber, and the filtrate was loaded onto combined silica and Florisil cartridge columns. The columns were washed with 10 mL of hexane–ethyl acetate (4 : 1) and eluted with 10 mL of ethyl acetate. The eluate was evaporated to dryness under reduced pressure at 35°C .

The residue was dissolved in 0.5 mL of freshly prepared 2.5% BSTFA ethyl acetate solution and kept at room temperature for 1 hr. This solution is hereinafter called "test solution for GC/MS".

Determination and confirmation of the proposed method

Patulin was determined by GC/MS in the selected ion monitoring (SIM) mode. The monitoring ions were m/z 226, 183 and 170, and quantification of patulin was based on the peak area of the m/z 226 ion. Positive samples were confirmed for patulin in the SCAN mode with the range of m/z 40–340. The mass spectrum of the peak with a similar retention time to patulin in the sample was compared with that of patulin standard. The conditions of GC/MS were as follows: column, BPX-35 capillary column (0.22 mm i.d. $\times$ 25 m, 0.25 μm film thickness, SGE Co., Cupertino, CA, USA); oven temperature, 80°C (2 min) $\rightarrow$ 150°C ($10^{\circ}\text{C}/\text{min}$ $\rightarrow$ 230°C ($5^{\circ}\text{C}/\text{min}$, 15 min); injection, 1 μL , splitless, 200°C ; detector, EI, 230°C ; electron energy, 70 eV; carrier gas, helium (constant pressure mode at 100 kPa).

Results and Discussion

Analysis of patulin by HPLC

Liquid chromatograms of the patulin standard and sample purified and analyzed by the AOAC official methods of analysis 995.10⁵⁾ are shown in Fig. 1. Many

interfering peaks followed after that of 5-hydroxymethylfurfural (HMF). Although this sample did not contain more than $1 \mu\text{g}$ patulin /kg, there was a peak

Fig. 1. Liquid chromatograms of standard and sample (A) Standard, $20 \mu\text{g}/\text{kg}$ equivalent in sample, (B) Sample, cleaned up by AOAC Official Methods for Analysis 995.10, patulin content: not more than $1 \mu\text{g}/\text{kg}$

Column: Puresil C_{18} , mobile phase: 0.095% perchloric acid (60%): CH_3CN ($97:3$), flow rate: $1.0 \text{ mL}/\text{min}$, detection: UV (276 nm), injection volume: $20 \mu\text{L}$.

with a similar retention time to patulin. The retention time of the peak was a little different from that of patulin, and at least 2 peaks overlapped. We found no way to determine patulin in this sample by HPLC. In some cases, precise quantification of patulin at a low level by HPLC was difficult. Moreover, it was difficult to confirm whether a peak with a similar retention time to patulin was patulin or not, because the UV spectra of patulin and HMF are very similar to each other.

Development of clean-up procedure

Even without clean-up after extraction, it was possible to determine patulin roughly in most apple juice samples by SIM mode analysis. However, injection of a solution containing a lot of impurities usually causes problems with the ion source of the MS detector and the capillary column. Some clean-up procedures are necessary for the sensitive and selective analysis of patulin.

1. Washing with sodium carbonate solution

Washing with 1.5% sodium carbonate solution after extraction is the clean-up step in the AOAC official method of analysis 995.10⁵⁾. This washing step had an insufficient effect for determining patulin at low level; it was not possible to confirm patulin at less than $20 \mu\text{g}/\text{kg}$ in sample by mass spectrometry. As patulin is also known to be unstable in an alkaline environment, we omitted this step.

2. Filtration in low-polarity condition and solid-phase extraction

In the AOAC official method of analysis 974.18⁵⁾, patulin was cleaned up by silica gel column chromatography. In the clean-up procedure, however, benzene, a very harmful solvent, was used. Therefore we developed a new clean-up step using other solvents.

When "sample extract" (ethyl acetate solution) was

Fig. 2. Mass chromatograms in scan mode of the sample after each clean-up step

(A) Blank sample after filtration, (B) Spiked sample ($50 \mu\text{g}/\text{kg}$) after filtration, (C) Blank sample after clean-up by solid-phase extraction, (D) Spiked sample ($50 \mu\text{g}/\text{kg}$) after clean-up by solid-phase extraction

Each test solution for GC/MS analysis contained 20 g equivalent of the sample.

The arrows show the retention time for the patulin derivative.

Fig. 3. Mass chromatogram of patulin TMS derivative in sample
Patulin content: 5.5 $\mu\text{g/kg}$ in sample.

directly applied to combined silica and Florisil cartridges, patulin passed through, while some impurities were held in the cartridges. The eluent was evaporated, and the residue was taken up in BSTFA solution and subjected to GC/MS analysis. Little improvement by this clean-up was observed in the mass chromatogram and mass spectra. In this study, therefore, hexane was added to the "sample extract" to hold patulin on the cartridges in hexane-ethyl acetate mixed solution containing more than 80% of hexane. By washing the cartridges with 10 mL of hexane-ethyl acetate (4:1), some impurities were eluted. Patulin was then completely eluted with 10 mL of ethyl acetate.

The effect of clean-up by this proposed method, filtration under non-polar conditions and solid-phase extraction, was checked. The mass chromatograms in the SCAN mode of the sample after each clean-up step are shown in Fig. 2.

When 2 mL of "sample extract" was evaporated to dryness and taken up in 0.5 mL of BSTEA solution, some residue was not dissolved and the solution was deeply colored and very clouded. Therefore we could not apply this test solution to GC/MS. When "sample extract" was mixed with nine volumes of hexane, significant amounts of insoluble substances were produced. After filtration under non-polar conditions, it was possible to dissolve the cleaned-up sample in 0.5 mL of BSTEA solution. This filtration was very effective for purification, removing polar substances. The filtration was more effective for clean-up in hexane-ethyl acetate (9:1) than in hexane-ethyl acetate (4:1), so hexane-ethyl acetate (9:1) was selected as the solvent for application to the cartridges. Without the solid-phase extraction, an interfering peak near the patulin derivative remained (Fig. 2A, B). However, this interfering peak was effectively removed by the solid-phase extraction (Fig. 2C, D), and this test solution for GC/MS was clear and colorless or slightly colored. After the filtration under non-polar conditions and the solid-phase extraction, patulin could be confirmed by mass

spectrometry down to the 5 $\mu\text{g/kg}$ level in samples.

Selection of column for GC/MS

Three kinds of capillary columns of moderately polar type, DB-17 (0.25 μm i.d. $\times$ 30 m, 0.25 μm film thickness, J & W), DB-1701 (0.25 μm i.d. $\times$ 30 m, 0.25 μm film thickness, J & W) and BPX-35 (0.22 μm i.d. $\times$ 25 m, 0.25 μm film thickness, BSE), were tested to determine and confirm patulin in samples. Although the determination of patulin in the SIM mode was possible with all tested columns, the best performance in the confirmation of patulin by mass spectrometry in the SCAN mode was achieved with the BPX-35 column; when analyzed with the other columns, some interfering fragment ion peaks derived from impurities disturbed the confirmation of patulin at low levels. The BPX-35 column was thus chosen for our method.

Determination and confirmation

The mass chromatogram of the sample is shown in Fig. 3. The monitored ions for the SIM mode were m/z 226, 183 and 170, based on their sensitivity and selectivity (Fig. 3). Although the sensitivity of m/z 183 was better than that of m/z 226, the best selectivity was obtained in the measurement at m/z 226, the molecular ion of patulin trimethylsilyl ether. Thus, it was decided to determine patulin by using the ion m/z 226. No interfering peak was found near this peak of patulin derivative.

Good linearity with a correlation coefficient of 0.9997 was obtained in the range of 40 to 8,000 ng/mL concentration of the solution injected into GC/MS, equivalent to 1 to 200 $\mu\text{g/kg}$ of patulin in samples. The limits of detection and quantification were 0.1 ($S/N=3$) and 1 $\mu\text{g/kg}$ ($S/N=30$) of patulin in samples, respectively.

As Fig. 4 shows, the mass spectrum of patulin trimethylsilyl ether in samples was in good agreement with that of the standard. Confirmation was possible at the concentration of 5 $\mu\text{g/kg}$ of patulin in samples and sometimes even at the level of 3 $\mu\text{g/kg}$. Confirmation from the mass spectrum obtained by GC/MS was con-

Fig. 4. Mass spectra of patulin derivative in sample (A) and standard (B)
Patulin content in the sample: 5.5 $\mu\text{g/kg}$.

Table 1. Recovery of Patulin Added to Apple Juice

Patulin added ($\mu\text{g/kg}$ in samples)	Mean recovery* (%)	CV (%)
10	93.4	3.8
20	94.9	2.4
50	99.8	1.8
500	100	1.6

* Values are means of five trials.

sidered to be reliable.

Recovery and effect of sample matrix

Different amounts (0.5, 1.0, 2.5 and 25 μg) of patulin were added to 50 g of 100% pure apple juice ($n=5$, each), and the samples were analyzed by the proposed method. The recovery and CV values ranged from 93.4 to 100% and 1.6 to 3.8%, respectively (Table 1).

The effect of the sample matrix on our GC/MS method was examined. No effect of the sample matrix on the sensitivity was observed. The recovery and CV values of the proposed method were sufficient for evaluation of apple juice samples.

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