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Development of Liquid Chromatography−Electrospray Mass Spectrometry for the Determination of Patulin in Apple Juice: Investigation of Its Contamination Levels in Japan

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Patulin is a mycotoxin produced by mainly *Penicillium* species, for example, *P. expansum*, and *Aspergillus* species. There are several reports of patulin contamination in apple juice. Last year, the Ministry of Health, Labour and Welfare of Japan set the maximum allowable level of patulin in apple juice at 50 ppb and decided that the measurement of patulin levels in apple juice products should be conducted. To this end, a simple, accurate, and selective analytical method for the detection of patulin at levels lower than 5 ppb, the detection limit, is desired. This paper reports the development of an analytical method that employs solid-phase extraction−liquid chromatography−mass spectrometry (SPE-LC-MS). When MS measurements were conducted with the selected ion monitoring (SIM) mode, the pseudomolecular ions at *m*/*z* 153 and 156 were used to monitor patulin and $^{13}C_3$-labeled patulin, respectively. The detection limit (S/N = 3) and the quantification limit (S/N = 10) of patulin at injection levels into LC-MS were 12.5 and 25 pg, respectively. However, when the actual sample was applied for the analysis based on the developed method including the sample preparation, the detection limit (S/N = 3) and quantification limit (S/N = 10) were 2.5 and 5 pg in sample, respectively. The calibration curve obtained for concentrations ranging from 5 to 500 ppb showed good linearity with a coefficient of determination (r^2) of 0.999. In addition, the recovery was >95% when an internal standard was used. The method was applied to the analysis of 76 apple juice samples from Japan, and as a result, patulin levels ranging from <1.0 to 45 ppb (detection frequency = 15/76) were detected. In this study, it was found that patulin was a greater contaminant in concentration/reduction than in "not from concentrate" apple juice.

KEYWORDS: Mycotoxin; patulin; apple juice; LC-MS; contamination

INTRODUCTION

Patulin, 4-hydroxy-4*H*-furo[3,2-*c*]pyran-2(6*H*)-one (**Figure 1**), is a mycotoxin produced by mainly *Penicillium* species, for example, *P. expansum*, and *Aspergillus* species (*1*−*4*). Recently, patulin in apple juice has become the focus of concern because of its adverse effects (*5*−*10*). Patulin inhibits the activity of numerous enzymes through its strong affinity for sulfhydryl groups (*8*). Moreover, it is capable of disturbing the mitochondrial and plasma membrane functions (*9*), and patulin has been shown to be immunosuppressive (*10*). The acute toxic effects of patulin on humans include nausea, vomiting, and other gastrointestinal trauma and accompanying kidney damage, and chronic exposure to patulin has been shown to induce the formation of cancerous tumors and to cause genetic mutations and embryonic developmental defects (*11*, *12*). When patulin-contaminated apples are processed for their juices, the patulin may be easily transferred into the juices (*13*, *14*). One source of human exposure is the ingestion of apple juice contaminated with patulin (*10*, *11*, *15*−*17*). As a consequence, the Ministry of Health, Labour and Welfare of Japan has proposed a maximum allowable level of patulin in apple juice products.

The Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives (JECFA) has established a provisional maximum tolerable daily intake (PMTDI) for patulin of 0.4 μg/kg of body weight/day (*11*). This limit is based on the results of evaluation of the no-observed-effect level (NOEL) in long-term toxicological studies using a 100-fold safety factor (*11*). Many countries have regulated the maximum allowable level of patulin in juice in the range of 20−50 ppb (*18*). The World Health Organization (WHO) considers patulin contamination in foods to be a serious problem and has set a maximum permissible concentration (MPC) of 50 ppb. For patulin in apple juice that is appropriately diluted for direct consumption by humans, the trend in some European countries is toward decreasing this limit to as low as 20 ppb.

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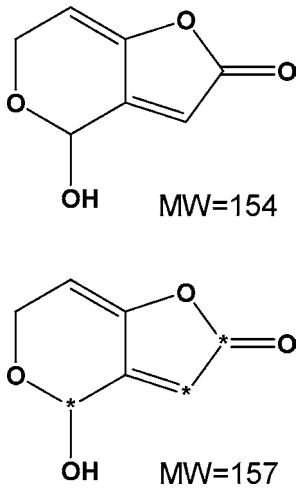

Figure 1. Chemical structures of patulin and $^{13}C_3$-labeled patulin. * indicates carbon 13-labeled.

Thus, there is a need to develop or modify existing analytical methods to realize a more sensitive determination of patulin in apple juice.

The methods of analysis of patulin in apple juice include gas chromatography (*19*−*21*), thin-layer chromatography (*22*), micellar electrokinetic capillary electrophoresis (*23*), and liquid chromatography (LC) (*9*, *10*, *24*, *25*). To evaluate the safety of apple juice, the level of patulin was determined by LC with ultraviolet (UV) detection (*26*−*28*) including the sample preparation with multiple liquid−liquid partition steps (*29*, *30*). Generally, patulin was difficult to separate with 5-hydroxymethylfurfural (HMF) performed by the LC-UV method; LC-MS detection with selected ion monitoring (SIM) mode was able to select only one ion. Therefore, its accuracy and selectivity was improved. Today, some European countries are decreasing this limit to as low as 20 ppb, so the method requires more sensitivity and accuracy. The method must be sufficiently sensitive to enable determination of the analyte in the parts per billion range. There are some literature studies that deal with the MS analysis of patulin (*9*, *30*, *31*); however, most of these methods include troublesome sample preparations. Recently, a multifunctional solid-phase column has been developed to replace the liquid−liquid partition steps. For example, Eisele et al. (*32*) carried out an easy and rapid solid-phase extraction (SPE) (syringe) method; however, patulin was detected by LC-UV. Therefore, in the present paper, we describe a method for the determination of patulin in apple juice using LC-MS with SPE for sample preparation. The method using the solid-phase column is simple and rapid: it takes 10 min to extract, isolate, and purify the patulin from apple juice. The test sample juice is applied to the cartridge, and patulin is eluted with ethanol and separated on a reversed-phase LC column.

MATERIALS AND METHODS

Reagents and Samples. Acetonitrile, HPLC grade, was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Water purified by a Milli-Q water purification system (Millipore, Bedford, MA) was used. Patulin was purchased from Sigma Aldrich Japan (Tokyo, Japan), and $^{13}C_3$-labeled patulin as the internal standard was purchased from Hayashi Pure Chemical Industries, Ltd. (Osaka, Japan).

Apple juice samples were obtained from various supermarkets and convenience stores in Japan.

Equipment. The LC-MS system was an Agilent LC-MSD Superior Line (Agilent Technologies, Palo Alto, CA) equipped with an electrospray ionization (ESI) source. A Mightysil RP-18 GP (2 × 250 mm, 5 μm) reversed-phase column and a Mightysil RP-18 GP guard column (2 × 5 mm, 5 μm) from Kanto Chemical Co., Ltd. (Tokyo, Japan) were used.

Standard Solution and Quantitative Procedure. Patulin stock solution was prepared in acetonitrile. Standard solutions of patulin were prepared in a water/acetonitrile = 1:1 (v/v) solution to cover the calibration range. Quantitative analysis was performed in the SIM mode to maximize sensitivity. Patulin concentration in each sample was calculated relative to the $^{13}C_3$-labeled patulin (**Figure 1**) standard added to the sample prior to direct analysis. Eight-point calibrations were performed for all samples with internal standards.

Sample Preparation. The pretreatment of beverages involves various extraction procedures. However, those procedures are difficult and require a large volume of organic solvent. In the present study, SPE was carried out with an extraction cartridge because it is very simple and uses a small volume of organic solvent. Apple juice samples were pretreated with three different SPE cartridges: *N*-vinylpyrrolidone with divinylbenzene polymer (OASIS-HLB; size = 200 mg/6 mL, Waters Co., Milford, MA), styrene−divinylbenzene polymer gel (GL-Pak PLS-2; size = 200 mg/6 mL, GL Sciences Co., Tokyo, Japan), and styrene−divinylbenzene with *N*-methacrylate (Aqusis PLS-3; size = 200 mg/6 mL, GL Sciences Co.). A solid-phase cartridge was placed on a vacuum manifold and conditioned with 10 mL of methanol followed by 10 mL of Milli-Q water prior to extraction. A sample was applied to the cartridge and allowed to flow through it under vacuum. After the sample had passed through the cartridge, it was washed with 10 mL of Milli-Q water under vacuum. The cartridge was aspirated under vacuum until most of the water was removed. Then, 5 mL of methanol was added at a low flow rate to elute the compounds that were retained on the cartridge.

The solution was evaporated to dryness under a stream of nitrogen at 40 °C. Then, the sample was adjusted with 4 mL of methanol and filtered through a 0.45 μm filter. The 5 μL sample solution was injected into the LC-MS system.

To confirm the utility of the internal standard, we added the $^{13}C_3$-labled patulin in apple juice before the sample preparation with SPE. Three kinds of solid-phase cartridges were compared; 100 ppb of patulin and $^{13}C_3$-labled patulin were added to clear apple juice, similarly, followed by SPE and patulin with internal standard detection, and, finally, recoveries were compared.

Chromatographic and MS Conditions. A sample volume of 5.0 μL was injected. LC separation was carried out using water (mobile phase A) and acetonitrile (mobile phase B). The gradient profile was as follows: 0 min in 100% mobile phase A, followed by 0−30 min of a linear decrease from 100 to 0% mobile phase A. The flow rate was 0.2 mL/min. The working conditions for ESI-MS were as follows: the drying nitrogen gas temperature was set at 350 °C, and the gas was introduced into the capillary region at a flow rate of 12 L/min; the capillary was held at a potential of 3500 V relative to the counter electrode in the negative-ion mode. The fragmentor voltage was 60 V for patulin and the internal standard during the chromatographic run. When the MS was conducted in the SIM mode, *m*/*z* 153 and 156 ions were detected and designated the $[M - H]^-$ ions of patulin and $^{13}C_3$-labeled patulin, respectively.

RESULTS AND DISCUSSION

Comparison of Recovery with SPE Cartridge. SPE with the reversed-phase mode cartridges was examined in terms of recovery. The recovery of patulin was examined using two apple juice samples (clear and cloudy) spiked with patulin standard solution. The extractions using the SPE cartridges were performed as described above. The recovery (100 ppb spiked) of patulin by the three SPE cartridges is shown in **Figure 2**. The Aqusis PLS-3 cartridge showed the highest recovery for samples spiked with patulin. Thus, we decided to use this cartridge for the simple and selective pretreatment of patulin in apple juice samples. The recovery and the relative standard deviation (RSD) for the clear and cloudy apple juice samples are shown in **Table 3**. However, a more accurate method was required to determine

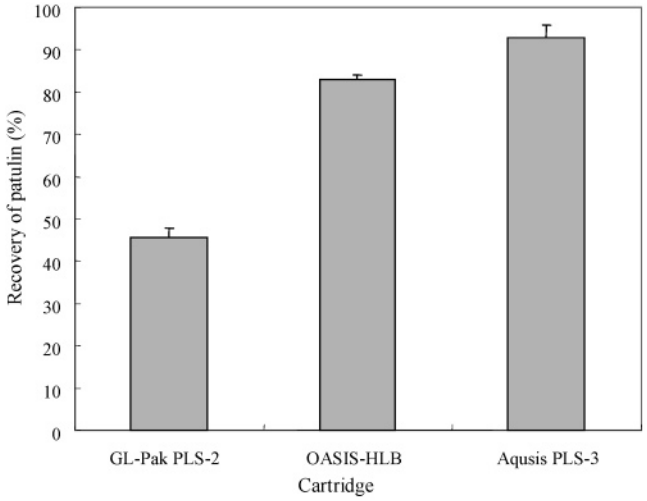

Figure 2. Comparison of recovery of patulin in apple juice using three SPE cartridges. Apple juice was spiked with 100 ppb of patulin and internal standard. Each plotted column is the mean average of recovery with triplicate analyses ($n = 3$). The error bar represents the standard deviation (SD).

Table 1. Recovery of Patulin in Apple Juice Samples Spiked with Patulin ($n = 3$)

	spiked concn (ppb)	recovery (%)	RSD (%)
cloudy	100	92.8	3.1
	10	101.2	7.1
clear	100	76.1	3.8
	10	114.4	10.8

Table 2. Recovery Test of Patulin in Apple Juice Samples to Which an Internal Standard Was Added ($n = 6$)

	spiked concn (ppb)	recovery (%)	RSD (%)
cloudy	100	99.6	1.5
	10	97.1	11.2
clear	100	96.6	3.2
	10	98.3	3.0

the level of patulin in commercially available apple juice. Therefore, the proposed SPE method was improved with an internal standard. **Table 1** shows the recovery of patulin in apple juice samples to which an internal standard was added. The recovery of patulin was >75% without the internal standard but was improved to >95% when an internal standard was added.

Determination of Patulin by LC-MS System. Using ESI-MS with flow-through injection analysis of the standard solutions, *m/z* 153 and 156 ions were observed as the main peaks for patulin $[M - H]^-$ and $^{13}C_3$-labeled patulin $[M - H]^-$, respectively (**Figure 3**). The corresponding SIM chromatograms are shown in **Figure 4**. One of the most important parameters affecting LC-MS for compound determination was the fragmentor voltage. The optimum fragmentor voltage was found to be 60 V (**Figure 5**). A high detection limit of ~10 times or more was acquired compared to that of a UV detector (detection wavelength = 276 nm). The detection limit (S/N = 3) and the quantification limit (S/N = 10) of patulin (5 μL injected) were 2.5 and 5 pg (in sample), respectively. For the quantitation of patulin in apple juices, the peak ratio of patulin to $^{13}C_3$-labeled patulin, the stable isotopically labeled internal standard, was calculated. A calibration curve was obtained for the peak ratio versus patulin concentration using HP ChemStation software from Agilent Technologies. A linear fit with a coefficient of determination (r^2) of 0.999 was observed for the SIM signal from 5 to 500 ppb. As the concentration of patulin in commercial apple juice is relatively low, the calibration curve for the low-concentration region is shown in **Figure 6**. The recovery of the proposed method was >95% ($n = 6$) using an internal standard.

Table 3. Levels of Patulin in Commercial Apple Juices in Japan[a]

sample	detection level (ppb)	manufacture	type	sample	detection level (ppb)	manufacture	type
1	ND	clear	D	39	8.6	clear	conc
2	ND	clear	D	40	5.7	clear	conc
3	ND	clear	D	41	3.2	clear	conc
4	ND	cloudy	D	42	1.9	clear	conc
5	ND	cloudy	D	43	ND	clear	conc
6	ND	cloudy	D	44	ND	clear	conc
7	ND	cloudy	D	45	ND	clear	conc
8	ND	cloudy	D	46	ND	clear	conc
9	ND	cloudy	D	47	ND	clear	conc
10	ND	cloudy	D	48	4.4	cloudy	conc
11	ND	cloudy	D	49	ND	cloudy	conc
12	21.4	–	D	50	ND	cloudy	conc
13	5.0	–	D	51	ND	cloudy	conc
14	4.5	–	D	52	1.4	blended	conc
15	ND	–	D	53	ND	blended	conc
16	ND	–	D	54	ND	blended	conc
17	ND	–	D	55	10.0	–	conc
18	ND	–	D	56	4.3	–	conc
19	ND	–	D	57	3.6	–	conc
20	ND	–	D	58	1.5	–	conc
21	ND	–	D	59	ND	–	conc
22	ND	–	D	60	ND	–	conc
23	ND	–	D	61	ND	–	conc
24	ND	–	D	62	ND	–	conc
25	ND	–	D	63	ND	–	conc
26	ND	–	D	64	ND	–	conc
27	ND	–	D	65	ND	–	conc
28	ND	–	D	66	ND	–	conc
29	ND	–	D	67	ND	–	conc
30	ND	–	D	68	12.3	clear	–
31	ND	–	D	69	ND	clear	–
32	ND	–	D	70	ND	cloudy	–
33	ND	–	D	71	ND	cloudy	–
34	ND	–	D	72	ND	blended	–
35	ND	–	D	73	45.6	–	–
36	ND	–	D	74	ND	–	–
37	ND	–	D	75	ND	–	–
38	ND	–	D	76	ND	–	–

[a] In the "type" and "manufacture" columns, unspecified juice is shown by "–". Moreover, in the "type" column, "D" indicates "directly produced" and "conc" indicates "from concentrate" apple juice. ND < 1 ppb.

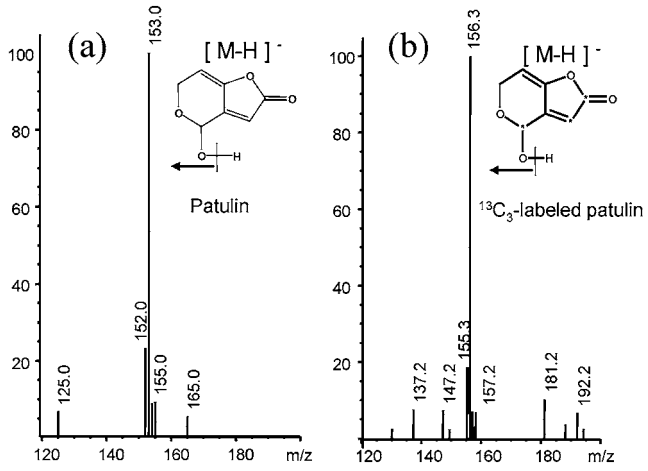

Figure 3. Mass spectra of patulin and its internal standard. Mass spectra were obtained after 5 ng of patulin or $^{13}C_3$-labeled patulin was injected.

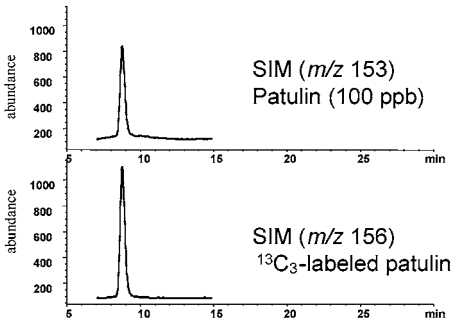

Figure 4. Selected ion monitoring chromatograms of patulin (*m*/*z* 153) and $^{13}C_3$-labeled patulin as internal standard (*m*/*z* 156). The chromatogram was obtained with 100 ppb of patulin standard, and 0.5 ng of patulin was injected.

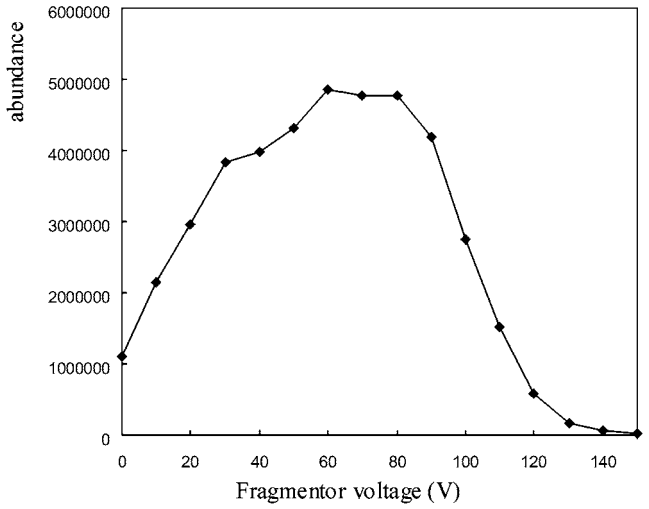

Figure 5. Optimization of fragmentor voltage.

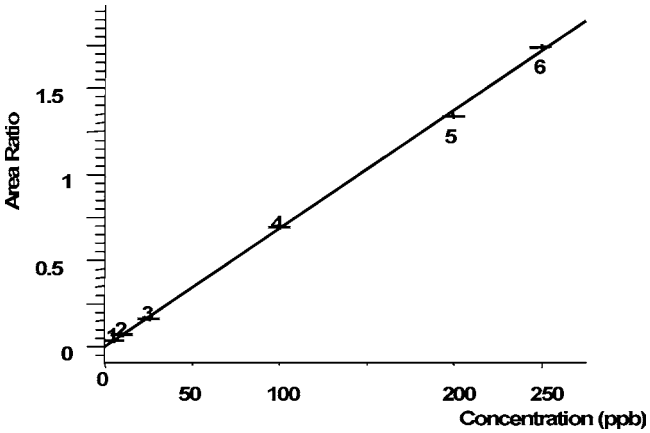

Figure 6. Calibration curve of patulin with internal standard.

Investigation of Patulin in Commercially Available Apple Juice in Japan. This method was applied to the analysis of 76 apple juice samples obtained in Japan, 14 of which were clear, 14 cloudy, 4 blended, and 44 of no specific type. The manufacturing method was either "from concentrate" or "directly produced", which was prepared without any concentration process during the manufacturing. Of the 76 samples, 38 were directly produced apple juices, 29 from concentrate apple juices, and the rest, unspecified. As a result, patulin at levels ranging from <1.0 to 45 ppb (detection frequency = 15/76) was detected from the apple juice samples (**Table 2**). Comparing the detection frequency in terms of manufacturing juices, patulin was detected in directly produced and from concentrate apple juices at frequencies of 3/38 and 10/29, respectively. In Japan, more patulin in the from concentrate juice than from the directly produced juice was detected. In addition, when the detection frequencies in terms of juice type were compared, patulin was detected in clear, cloudy, and blended apple juices at frequencies of 5/14, 1/14, and 1/4, respectively. As a result, we concluded that the concentration of patulin in apple juice was not influenced by the juice type.

Conclusion. In the present paper, the detection frequency was higher because the proposed method has high sensitivity. The LC-MS with SPE method was developed to determine patulin in apple juice. This is the first surveillance, which determined patulin in apple juices in Japan with an SPE-LC-MS method. It was simpler than the conventional method and determined patulin in high sensitivity. Therefore, the proposed method was very useful for the determination of patulin in apple juice.

LITERATURE CITED

(1) Lovett, J.; Thompson, R. G., Jr. Patulin production by species of *Aspergillus* and *Penicillium* at 1.7, 7.2 and 12.8 °C. *J. Food Prot.* **1978**, *41*, 195−197.

(2) Northolt, M. D.; van Egmond, H. P.; Paulsch, W. E. Patulin production by some fungal species in relation to water activity and temperature. *J. Food Prot.* **1978**, *41*, 885−890.

(3) Roland, J. O.; Beuchat, L. R. Biomass and patulin production by Byssochlamys nivea in apple juice as affected by sorbate, benzoate, SO_2 and temperature. *J. Food Sci.* **1984**, *49*, 402−406.

(4) Andersen, B.; Smedsgaard, J.; Frisvad, J. C. *Penicillium expansum*: consistent production of patulin, chaetoglobosins, and other secondary metabolites in culture and their natural occurrence in fruit products. *J. Agric. Food Chem.* **2004**, *52*, 2421−2428.

(5) Wichmann, G.; Herbarth, O.; Lehmann, I. The mycotoxins citrinin, gliotoxin, and patulin affect interferon gamma rather than interleukin-4 production in human blood cells. *Environ. Toxicol.* **2002**, *17*, 211−218.

(6) Sugiyanto, J.; Inoue, M.; Oda, S.-I.; Takagishi, Y.; Yamamura, H. Teratogenicity of patulin, a mycotoxin, in mice. *Environ. Med.* **1993**, *37*, 43−46.

(7) Burghardt, R. C. Patulin-induced cellular toxicity: A vital fluorescence study. *Toxicol. Appl. Pharmacol.* **1992**, *112*, 235−244.

(8) Wouter, M. F. A.; Speijers, G. J. A. *Patulin. Toxicological Evaluation of Certain Food Additives and Contaminants*; World Health Organization: Geneva, Switzerland, 1996; pp 337−402.

(9) Sewram, V.; Nair, J. J.; Nieuwoudt, T. W.; Leggott, N. L.; Shephard, G. S. Determination of patulin in apple juice by high-performance liquid chromatography-atmospheric pressure chemical ionization mass spectrometry. *J. Chromatogr. A* **2000**, *897*, 365−374.

(10) Food and Agriculture Organization/World Health Organization. *Joint FAO/WHO Expert Committee on Food Additives*, 56th Meeting, Feb 6−15, 2001; WHO: Geneva, Switzerland, 2001; pp 1−33 (http://www.fao.org/es/ESN/Jecfa/jecfa56.pdf).

(11) World Health Organization. Evaluation of certain food additives and contaminants. *44th Report of the Joint FAO/WHO Expert Committee on Food Additives*; Technical Report Series 859; Geneva, Switzerland, 1995; pp 36−38.

(12) World Health Organization, International Agency for Research on Cancer. IARC Monograph on the Evaluation of the Carcinogenic Risk of Chemicals to Humans 40; IARC: Lyon, France, 1986; pp 92−93.

(13) Drusch, S.; Ragab, W. Mycotoxins in fruits, fruit juices, and dried fruits. *J. Food Prot.* **2003**, *66*, 1514−1527.

(14) Yurdun, T.; Omurtag, G. Z.; Ersoy, Ö. Incidence of patulin in apple juices marketed in Turkey. *J. Food Prot.* **2001**, *64*, 1851−1853.

(15) Forbito, P. R.; Babsky, N. E. Rapid liquid chromatographic determination of patulin in apple juice. *J. Assoc. Off. Anal. Chem.* **1985**, *68*, 950−951.
(16) Stray, H. High-pressure liquid chromatographic determination of patulin in apple juice. *J. Assoc. Off. Anal. Chem*. **1978**, *61*, 1359−1362.
(17) Vural G.; Jale, A. Long-term survey of patulin in apple juice concentrates produced in Turkey. *Food Addit. Contam*. **2000**, *17*, 933−936.
(18) Food and Agriculture Organization of the United Nations. *FAO Worldwide Regulations for Mycotoxins for 1995*; Food and Nutrition Paper 64; FAO: Rome, Italy, 1996.
(19) Ralls, J. W.; Lane, R. M. Examination of cider vinegar for patulin using mass spectrometry. *J. Food Sci*. **1977**, *42*, 1117−1119.
(20) Suzuki, T.; Fujimoto, Y.; Hoshino, Y.; Tanaka, A. Simultaneous determination of patulin and penicillic acid in grains by gas chromatographic method. *Agric. Biol. Chem*. **1974**, *38*, 1259−1260.
(21) Tarter, E. J.; Scott, P. M. Determination of patulin by capillary gas chromatography of the heptafluorobutyrate derivative. *J. Chromatogr*. **1991**, *538*, 441−446.
(22) Betina, V. Thin-layer chromatography of mycotoxins. *J. Chromatogr*. **1985**, *15*, 211−276.
(23) Tsao, R.; Zhou, T. Micellar electrokinetic capillary electrophoresis for rapid analysis pf patulin in apple cider. *J. Agric. Food Chem*. **2000**, *48*, 5231−5235.
(24) MacDonald, S.; Long, M.; Gilbert, J. Liquid chromatographic method for determination of patulin in clear and cloudy apple juices and apple puree: Collaborative study. *J. Assoc. Off. Anal. Chem*. **2000**, *83*, 1387−1394.
(25) Türkan, Y.; Gülden, Z. O.; Ömer, E. Incidence of patulin in apple juices marketed in Turkey. *J. Food Prot*. **2001**, *64*, 1851−1853.
(26) Trucksess, M.; Tang, Y. Solid-phase extraction method for patulin in apple juice and unfiltered apple juice. *J. Assoc. Off. Anal. Chem*. **1999**, *82*, 1109−1113.
(27) Tangni, E. K.; Theys, R.; Mignolet, E.; Maudoux, M.; Michelet, J. Y.; Larondelle, Y. Patulin in domestic and imported apple-based drinks in Belgium: occurrence and exposure assessment. *Food Addit. Contam*. **2003**, *20*, 482−489.
(28) Ritieni, A. Patulin in Italian commercial apple products. *J. Agric. Food Chem*. **2003**, *51*, 6086−6090.
(29) Brause, A.; Trucksess, M.; Thomas, F.; Page, S. Determination of patulin in apple juice by liquid chromatography: collaborative study. *J. Assoc. Off. Anal. Chem*. **1996**, *79*, 451−456.
(30) Rychlik, M.; Schieberle, P. Quantification of the mycotoxin patulin by a stable isotope dilution assay. *J. Agric. Food Chem*. **1999**, *47*, 3749−3755.
(31) Sheu, F.; Shyu, Y. T. Analysis of patulin in apple juice by diphasic dialysis extraction with in situ acylation and mass spectrometric determination. *J. Agric. Food Chem.* **1999**, *47*, 2711−2714.
(32) Eisele, T. A.; Gibson, M. Z. Syringe-cartridge solid-phase extraction method for patulin in apple juice. *J. AOAC Int.* **2003**, *86*, 1160−1163.

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