Report

Inter-laboratory Study for Validation of a Japanese Official Analytical Method for Determination of Patulin in Apple Juice

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Yoshiko SUGITA-KONISHI^{*1, †}, Toshitugu TANAKA^{*2}, Yoshitugu SUGIURA^{*2},
Setsuko TABATA^{*3}, Masahiro NAKAJIMA^{*4}, Hiroyuki SAKURAI^{*5}, Yoko NAKAIE^{*6},
Koichi SATO^{*7}, Yusuke KITANI^{*7}, Kazuhiro FUJITA^{*8}, Sachiko HAYASHI^{*8}, Tayoshi IIZUKA^{*9},
Yoshinori HIRAKAWA^{*9}, Naoki MOCHIZUKI^{*10}, Mariko HOSHINO^{*10}, Yoshirou SATO^{*11},
Naomi TAKAHASHI^{*11} and Kosuke TAKATORI^{*1}

(^{*1} National Institute of Health Sciences: 1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan;

^{*2} Kobe Institute of Health: 4-6, Minatojima-nakamachi, Chuo-ku, Kobe 650-0046, Japan;

^{*3} Tokyo Metropolitan Institute of Public Health: 3-24-1, Hyakunin-cho,
Shinjuku-ku, Tokyo 169-0073, Japan;

^{*4} Nagoya City Public Health, Research Institute: 1-11, Hagiya, Mizuho-ku,
Nagoya 467-8615, Japan;

^{*5} Yokohama Quarantine Station: 107-8, Nagahama, Kanazawa-ku, Yokohama 236-0011, Japan;

^{*6} Kobe Quarantine Station: 1-1, Toyahamacho, Kobe, Hyogo 230-0862, Japan;

^{*7} IAA Center for Food Quality, Labeling and Consumer Services: 2-1,
Shintoshin, Chuo-ku, Saitama 330-9731, Japan;

^{*8} Japan Food Research Laboratories, Nagoya Branch: 4-5-13, Otu,
Naka-ku, Nagoya 460-0011, Japan;

^{*9} Japan Food Research Laboratories, Japan Inspection Association of Food and
Food Industry Environment: 3-7-4, Kyobashi, Chuo-ku, Tokyo 104-0031, Japan;

^{*10} Asahi Breweries, Ltd.: 1-1-21, Midori, Moriya-shi, Ibaraki 302-0106, Japan;

^{*11} Meiji Dairies Corporation: 540, Naruta, Odawara, Kanagawa
250-0862, Japan; † Corresponding author)

To validate a modified version of AOAC official method of analysis 995.10 as an official standard in Japan for determination of patulin in apple juice, an inter-laboratory study was performed in 11 laboratories using a non-contaminated sample, 2 naturally contaminated samples and 2 spiked samples of apple juice. For naturally contaminated apple juices, the relative standard deviations for repeatability and reproducibility were 3.2, 7.1% and 10.0, 21.7%, respectively. HORRAT values were 0.4, 0.9. The average recovery of patulin from spiked sample was 83.7%. The limit of quantification was calculated as 10 µg/kg. From these results, the method was thought to be suitable as an official standard for determination of patulin in apple juice in Japan.

Key words: apple juice; patulin; official method; HPLC; inter-laboratory study; validation

Introduction

Patulin is a mycotoxin which contaminates fruit juices, especially apple juice. It is mainly produced by *Penicillium expansum*. The toxicity of patulin was evaluated in 1996 by the Joint FAO/WHO Expert Committee on Food Additives (JECFA)⁽¹⁾, which concluded that the provisional maximal tolerable daily intake of patulin is 0.4 µg/kg body weight/day.

The Codex committee has recommended a maximum level of patulin in apple juice of 50 µg/kg and many countries have established a standard for patulin. In Japan, the Ministry of Health, Labour and Welfare established a limit of 50 µg/kg in 2004⁽²⁾. To enforce this,

an official method for determination of patulin in apple juice should be established.

Several analytical methods for measuring levels of patulin have been developed, based on high-performance liquid chromatography (HPLC)^{(3), (4)}, GC-MS^{(5), (6)}, LC-MS⁽⁷⁾, etc. Among them, two procedures using ethyl acetate for extraction and sodium carbonate for cleaning-up before HPLC have been published, AOAC official methods 995.10⁽³⁾ and 2000.02⁽⁴⁾, and are used in many countries to determine the concentration of patulin in apple juice. However less organic solvent and time are required for 995.10 than 2000.02. Therefore, in this study, an HPLC-based analytical procedure modified from method 995.10 was validated as an

official standard for determination of patulin in apple juice in an inter-laboratory study involving 11 laboratories.

Materials and Methods

Standard and reagents

A patulin standard was purchased from Sigma Chemicals Co. (St. Louis, MO, USA). The patulin (5 mg) was weighed into a 50 mL volumetric flask. The toxin was dissolved in ethyl acetate as a stock solution and stored in a freezer at -20°C . One milliliter of this stock solution was evaporated to dryness under a stream of nitrogen gas, and the residue was immediately dissolved in 10 mL of ethanol (*ca.* $10\text{ }\mu\text{g/mL}$). The UV absorbance was measured, and the concentration of patulin was calculated. Two more portions of the stock solution of patulin were appropriately diluted with acetonitrile to prepare spiked solutions (246.7 ng/mL and 101.2 ng/mL) in the laboratory (National Institute of Health Sciences). Organic solvents, such as ethyl acetate and acetonitrile of HPLC grade, water of HPLC grade, and reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Preparation of samples

Clear and clouded samples of apple juice naturally contaminated with patulin, which were used in the Food Analytical Performance Assessment Scheme (FAPAS) Program carried out in 2003, were purchased from Central Science Laboratories (York, UK). The assigned concentrations of patulin in these samples were $22.5\text{ }\mu\text{g/kg}$ in the clear apple juice and $49.8\text{ }\mu\text{g/kg}$

in the clouded apple juice, according to the FAPAS report. Commercial clear type apple juice in which the patulin content was found to be below the limit of quantification using procedure 995.10, the official AOAC method of analysis³⁾, was selected as a blank.

Fortification procedure

For recovery determinations, a 1 mL aliquot of each spiked solution was taken into a 30 to 50 mL centrifuge tube and evaporated under nitrogen gas to eliminate acetonitrile. Five milliliters of non-contaminated apple juice was immediately added to individual tubes, and mixed well (equivalent to fortification levels of $49.3\text{ }\mu\text{g/kg}$ and $20.2\text{ }\mu\text{g/kg}$ patulin, respectively). One hour later, the toxin was extracted and clean-up was carried out according to the protocol described in this paper.

Protocol used by participants

Scheme 1 shows the protocol for the analytical procedure (Japanese official method) modified from method 995.10. Briefly, 5 g of apple juice was placed in a 30 to 50 mL centrifuge tube, and 10 mL of ethyl acetate was added. After 1 min of shaking, the ethyl acetate layer was transferred to a new centrifuge tube. This extraction procedure was repeated three times. These ethyl acetate extracts were combined in the centrifuge tube, 2 mL of 1.5% sodium carbonate solution was added, and the tube was shaken vigorously for 10–20 sec. The ethyl acetate layer was transferred to a clean tube, and the lower aqueous layer was extracted with 5 mL of ethyl acetate. Then the ethyl acetate layer was added to the tube and evaporated to dryness under nitrogen gas.

Scheme 1. Protocol of the method for determination of patulin in apple juice validated with an inter-laboratory study

Table 1. HPLC Conditions

Column: ODS 100–250 mm×4.6 mm i.d. (3–5 μ m)
Column temp.: 40°C
Flow rate: 1.0 mL/min
Wavelength: 276 nm (290 nm)
Injected volume: 40 μ L
Mobile phase: Acetonitrile–water (4:96)

The residue was dissolved in 1 mL of acetic acid (approximately 0.01%, pH 3.6–4.0). After filtration, the sample was subjected to HPLC analysis.

HPLC conditions

HPLC conditions are listed in Table 1. The analytical ODS column (100–250 mm×4.6 mm i.d., 3–5 μ m) was kept at 40°C and the mobile phase was acetonitrile–water (4:96 v/v), delivered at a flow rate of 1.0 mL/min. Detection was performed with a UV detector at a wavelength of 276 nm.

Inter-laboratory study

For validation of the official method, an inter-laboratory study was carried out using 5 materials (two naturally contaminated apple juices, two spiked apple juices and a blank) according the protocol of the International Union of Pure and Applied Chemistry (IUPAC)⁸. Eleven laboratories participated in the inter-laboratory study, that is, National Institute of Health Sciences, Kobe Institute of Health, Nagoya City Public Health Research Institute, Tokyo Metropolitan Insti-

tute of Public Health, Yokohama Quarantine Station, Kobe Quarantine Station, IAA Center for Food Quality, Labeling and Consumer Services, Japan Food Research Laboratories, Nagoya Branch, Japan Inspection Association of Food and Food Industry Environment, Asahi Breweries, Ltd. and Meiji Dairies Corporation.

Statistics

The raw data sent by the participants were subjected to statistical tests (Cochran, Grubbs and Grubbs paired tests) according to the AOAC guide⁹. First the suspect and outlying data between blind duplicates (Corhran test) and between laboratory means (Grubbs test) were analyzed. Then, the parameters of precision, which are the inter-laboratory relative standard deviations for repeatability (RSD_r) and for reproducibility (RSD_R), were deduced.

Results and Discussion

The procedure used in this study was a modified version of AOAC official method of analysis 995.10 in which tetrahydrofuran was omitted from the mobile phase, because it is known to be corrosive.

Five samples were used in this inter-laboratory study, non-contaminated, clear type naturally contaminated, clouded type naturally contaminated and two concentrations of spiked apple juice. The number of materials satisfied the minimum for a quantitative study using the collaborative study protocol of IUPAC. The results received from the 11 laboratories regarding the concentration of patulin in naturally contaminated apple juice

Table 2. Inter-laboratory Results (Blind Duplicate) for Determination of Patulin in Apple Juice by the Method Modified from the AOAC Official Method 995.10

Laboratory codes	Non-contaminated apple juice (μ g/kg)		Naturally contaminated apple juice (μ g/kg)				Recovery (%)			
			Clear type		Cloud type		Spiked 1 (49.3 μ g/kg)		Spiked 2 (20.2 μ g/kg)	
	Assay 1	Assay 2	Assay 1	Assay 2	Assay 1	Assay 2	Assay 1	Assay 2	Assay 1	Assay 2
1	ND	ND	21.7	23.1	69.6	68.4	86.8	94.1	96.0	78.2
2	ND	ND	18.8 (C)	11.4 (C)	44.4	38.7	80.3	83.1	97.0	86.1
3	ND	ND	19.9	19.7	65.1	64.5	75.2	81.7	79.7	72.2
4	ND	ND	20.4	20.5	53.2	53.0	95.5 (G)	98.5 (G)	91.5	93.0
5	ND	ND	18.8	19.2	46.0	48.2	87.2	86.2	83.6	78.2
6	ND	ND	19.9	19.9	73.2	62.8	89.6	92.4	97.5	93.5
7	ND	ND	19.5	18.1	42.5	39.3	88.6	77.4	81.1	73.2
8	ND	ND	18.5	18.4	44.3	41.7	75.6	78.2	72.7	81.6
9	ND	ND	23.0	19.3	44.5	54.8	79.9	95.1	86.1	76.2
10	ND	ND	14.8	16.4	38.4	43.3	39.9 (G)	54.7 (G)	68.3	59.9
11	ND	ND	20.7	21.3	49.5	48.9	83.1	84.1	87.1	95.0
Number of laboratories	10		11		9		11			
Number of outliers laboratories	1		0		2		0			
Overall mean or recovery (μ g/kg or %)	19.5		51.6		84.4 (%)		83.0 (%)			
Repeatability SD [Sr, μ g/kg]	0.6		3.7		5.1		6.4			
Repeatability relative SD [RSD _r , %]	3.2		7.1		6.1		7.7			
Repeatability value [t (2.8×Sr)]	1.8		10.2		14.4		18.0			
Reproducibility SD [SR, μ g/kg]	2.0		11.2		6.1		10.4			
Reproducibility relative SD [RSD _R , %]	10.0		21.7		7.3		12.5			
Reproducibility value [R (2.8×SR)]	5.5		31.3		17.2		29.2			
HORRAT	0.4		0.9		0.3		0.4			

Abbreviations: C=Cochran outlier, G=Grubbs outlier, ND=Not determined

and the rate of recovery are listed in Table 2.

Performance parameters are shown for 1 case of an outlier in clear naturally contaminated apple juice and for 2 cases of an outlier in spiked juice. For non-contaminated apple juice, no laboratory detected patulin at a concentration above the level of the limit of detection. For the naturally contaminated juices, the RSD_r was 3.2% for clear type and 7.1% for clouded type, calculated without outliers. The RSD_R was 10.0% for clear type and 21.7% for clouded type. The HORRAT value was 0.4 for clear type and 0.9 for clouded type.

Tests were also carried out to determine the recovery from the samples spiked with 49.3 $\mu\text{g/kg}$ or 20.2 $\mu\text{g/kg}$ of patulin. For the two spiked samples, the mean recovery rate was 83.7%, calculated without outliers. For mycotoxins, a method to be recognized as an official method is required to have a recovery in the range of 70–110%¹⁰⁾. Hence, the rate of recovery obtained in the inter-laboratory study was acceptable. The RSD_r values of the samples spiked with 49.3 $\mu\text{g/kg}$ and 20.2 $\mu\text{g/kg}$ of patulin were 6.1% and 7.7% and the RSD_R values were 7.3% and 12.5%, respectively. The HORRAT value was 0.3 for the sample spiked with 49.3 $\mu\text{g/kg}$ of patulin and 0.4 for that spiked with 20.2 $\mu\text{g/kg}$ of patulin, respectively.

In the calibration curve, good linearity with a correlation coefficient of >0.9997 was obtained in the range of 50–500 ng/mL of patulin standard solution. From the $S/N (=3)$ value, the limit of detection was calculated as 3.0 $\mu\text{g/kg}$ and the limit of quantification was calculated as 10.0 $\mu\text{g/kg}$ from the minimum dose obtained from the linear calibration curve. Since the maximum level of patulin allowable in apple juice in Japan is 50 $\mu\text{g/kg}$, this method is effective to 1/5 of the maximum level.

Brause *et al.*¹¹⁾ performed an international collaborative study using the method 995.10. In their case, the average recovery was 96%, and the RSD_r and the RSD_R ranged from 10.9 to 53.8% and from 15.1 to 68.8%, respectively. In our study, though the average recovery was lower than their results, satisfactory parameters of precision were obtained. Recently, Arranz *et al.*¹²⁾ reported an inter-laboratory study using a method that involves extraction with ethyl acetate, followed by a silica gel solid-phase extraction clean-up. The results showed that the method allows determination of patulin at 10 $\mu\text{g/kg}$ with sufficient precision. In future, if the maximum level of patulin in apple-based products intended for infants is set at 25 $\mu\text{g/kg}$ in Japan, as has been suggested, it will be necessary to validate a more precise method. However, as the maximum limit of patulin in apple juice is set at 50 $\mu\text{g/kg}$ at present, the method validated in this study can be adopted as the official standard in Japan.

Tabata *et al.*⁶⁾ pointed out that the UV spectrum of the interfering peak close to patulin was very similar to that of patulin when a wavelength of 276 nm was used for detection. To distinguish patulin from the interfering peak, AOAC recommends using a non end-capped

HPLC column with a carbon load of 16% in both official methods. Further, our study revealed that the interfering peak was reduced¹³⁾ when UV detection was performed at 290 nm. Good linearity in the calibration curve was obtained at 290 nm as well as at 276 nm, though the sensitivity of detection was declined 30% lower in comparison with that at 276 nm. Therefore, close attention to selecting the HPLC column and a wavelength of 290 nm are recommended for the official Japanese method.

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