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Food Chemistry xxx (2004) xxx–xxx

Food Chemistry

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Analysis of cyclopiazonic acid in corn and rice by a newly developed method

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Received 28 June 2004; received in revised form 10 September 2004; accepted 10 September 2004

Abstract

This paper describes an improved method for the detection of cyclopiazonic acid (CPA), an indole tetramic acid mycotoxin, that does not use chloroform. The method includes precipitation of protein with lead acetate, liquid–liquid partitioning with diethyl ether, and determination by HPLC with UV and a photodiode array detector. The quantification limit of the method was 25 ng CPA/g for both corn and rice. The average recoveries from corn spiked with 25, 50, 100 and 200 ng CPA/g were 64.7%, 68.5%, 74.6% and 75.4%, respectively, and from rice spiked with 25, 50, and 100 ng CPA/g were 51.4%, 70.4%, and 82.1%, respectively. The method was successfully applied to the analysis of corn samples from the Philippines and rice samples from Thailand. Out of 6 corn samples analyzed, one sample was contaminated with 76 ng/g of CPA. No rice sample was contaminated with CPA. This is the first report of the natural occurrence of CPA in corn from the Philippines.

Keywords: Cyclopiazonic acid; Corn; Rice; Clean-up; HPLC; Philippines; Thailand

1. Introduction

Cyclopiazonic acid (CPA, Fig. 1) is an indole tetramic acid mycotoxin first isolated from cultures of *Penicillium cyclopium* Westling (Holzapfel, 1968) and is known as a secondary metabolite produced by numerous species of *Penicillium* (Le Bars, 1979) and *Aspergillus* (Luk, Kobbe, & Townsend, 1977; Ohmomo, Sugita, & Abe, 1973). Interestingly, some strains of *Aspergillus flavus* produce, not only aflatoxins (AF) that have potent carcinogenic and mutagenic toxicity, but also CPA as major metabolites (Gallagher, Richard, Stahr, & Cole, 1978; Lee & Hagler, 1991; Martins & Martins, 1999). The toxicity of CPA in many animal species has been studied. CPA causes weight loss, diarrhea, degeneration and necrosis of the muscles and viscera, and convulsion and death in rodents (Morrissey, Norred, Cole, & Dorner, 1985; Purchase, 1971), birds (Dorner, Cole, Lomax, Gosser, & Diener, 1983), dogs (Nuehring, Rowland, Harrison, Cole, & Dorner, 1985), and swine (Lomax, Cole, & Dorner, 1984). CPA has been implicated in two acute mycotoxicoses in humans: 'Koudua poisoning', for which the kodo millet produced symptoms of giddiness and nausea in man (Rao & Husain, 1985) and 'Turkey X disease', for which CPA was considered to be responsible in addition to AF (Blount, 1961; Cole, 1986; Sargent, Sheridian, O'Kelly, & Carnaghan, 1961). Although no natural incidence of disease of domestic animals has been conclusively related to CPA, its toxicity has been experimentally revealed. Furthermore, fungi producing CPA are widespread in nature (Gallagher et al., 1978; Horn & Dorner, 1999), and the natural occurrence of CPA has been reported in corn (Urano, Trucksess, Beaver et al., 1992; Widiastuti, Maryam,

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doi:10.1016/j.foodchem.2004.09.017

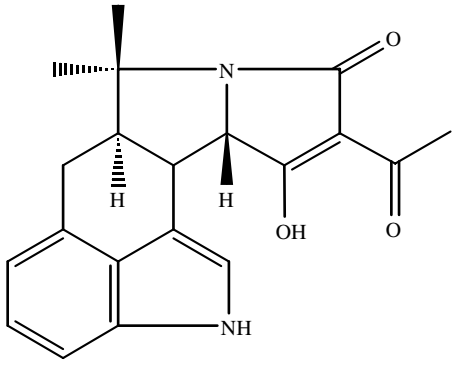

Fig. 1. Structure of cyclopiazonic acid.

Blaney, Stoltz, & Stoltz, 1988), peanuts (Lansden & Davidson, 1983; Urano, Trucksess, Beaver et al., 1992), cheese (Finoli, Vecchio, Galli, & Franzetti, 1999; Le Bars, 1979), millet (Rao & Husain, 1985), and various feeds and feedstuffs (Balachandran & Parthasarathy, 1996). Therefore, it is necessary to evaluate the CPA level in foods and feeds and address its relative risk to human and animal health.

Different methods, such as immunoassay (Hahnau & Weiler, 1991), capillary electrophoresis (Prasongsidh, Kailasapathy, Skurray, & Bryden, 1998), and chromatography (Lansden, 1984), to detect and quantify CPA, have been reported. Among these, liquid chromatographic methods including ligand exchange chromatography on a reversed-phase column (Lansden, 1984) normal-phase liquid chromatography on a silica gel column (Goto, Shinshi, Tanaka, & Manabe, 1987), and metal complexation chromatography on a reversed-phase column (Urano, Trucksess, Matusik, & Dorner, 1992) have been developed to quantify CPA in agricultural commodities. The HPLC method performed by Urano et al. (1992) is considerably sensitive and can detect concentrations as low as 50 ng/g of CPA in corn. However, there is difficulty in removing interference from the sample matrix, and most chemical methods that involve derivatization of the target compound and extensive clean-up procedures are time-consuming and complicated. Recently, a similar approach has been reported for simultaneous determination of CPA and tenuazonic acid in tomato products (Da Motta & Valente Soares, 2001). More recently, an amino-bonded silica phase separation with UV detection has been reported to be highly sensitive (Zambonin, Monaci, & Aresta, 2001). In most cases, toxic solvents such as chloroform and dichloromethane were used as extraction and clean-up solvents.

On the other hand, there have been very few investigations on the determination of CPA in naturally contaminated agricultural commodities from Asia. Human exposure to mycotoxins in staple foods, such as corn and rice, is an important issue in Asia due to the frequency of their intake. Therefore, this study was conducted to improve methods for the analysis of CPA in corn and rice from Southeast Asia without using toxic solvents such as chloroform.

2. Materials and methods

2.1. Chemicals and mycotoxin standards

All reagents were purchased from Wako Pure Chemical, Ltd. (Osaka, Japan) unless otherwise noted. Acetonitrile was of reagent grade and distilled in a glass apparatus before use. Diethyl ether was of analytical-reagent grade. A CPA standard was obtained from Sigma Co. (St. Louis, MO, USA). The stock solution of CPA in methanol (1 mg/ml) was prepared and stored at 4 °C in the dark. Working solutions were prepared by serial dilution with methanol:1 mM zinc sulfate (85:15, v/v).

2.2. Corn and rice samples

Corn grits (10 kg) and corn kernels (2 kg) from South Africa purchased from Okubo Denpun, Ltd. (Okayama, Japan) and polished rice (2 kg) from a local market (Kagawa, Japan) were used for clean-up and the recovery tests, respectively. For evaluation of the natural contamination of CPA in corn kernel, samples (more than 100 g each) were collected from the Philippines in 1997 and 1998, and rice samples (more than 100 g each) were obtained from Thailand in 2002. These samples were intended for human consumption. All samples were stored in zip-lock plastic bags at −20 °C until assayed. Samples were ground to fine powder using a Wonder blender (Iwaki Co. Ltd., Tokyo), and sieved through a 0.5-mm size sieve. These samples were previously analyzed for AF in our laboratory (Arim, 2000; Lipigorngoson, Ali, & Yoshizawa, 2003).

2.3. Extraction and clean-up

The schematic procedure for the analysis of CPA in corn and rice is summarized in Fig. 2. Ground samples (15 g each) were extracted with acetonitrile:1% sodium bicarbonate (7:3, v/v, 50 ml) and centrifuged at $1400 \times g$ for 5 min. The supernatant (30 ml) was mixed with 0.05 M lead acetate solution (60 ml) and filtered, using an Advantec GA-55 glass filter (Advantec Toyo Kaisya, Ltd., Tokyo, Japan). The filtrate (40 ml) was adjusted to pH 2 by adding 0.5 N hydrochloric acid solution (ca. 5.5 ml), and partitioned twice with diethyl ether (25 ml each). As an additional clean-up step, the combined diethyl ether extract (ca. 50 ml) was extracted twice with 5% sodium bicarbonate (20 ml each). The combined aqueous layer (ca. 40 ml) was acidified to

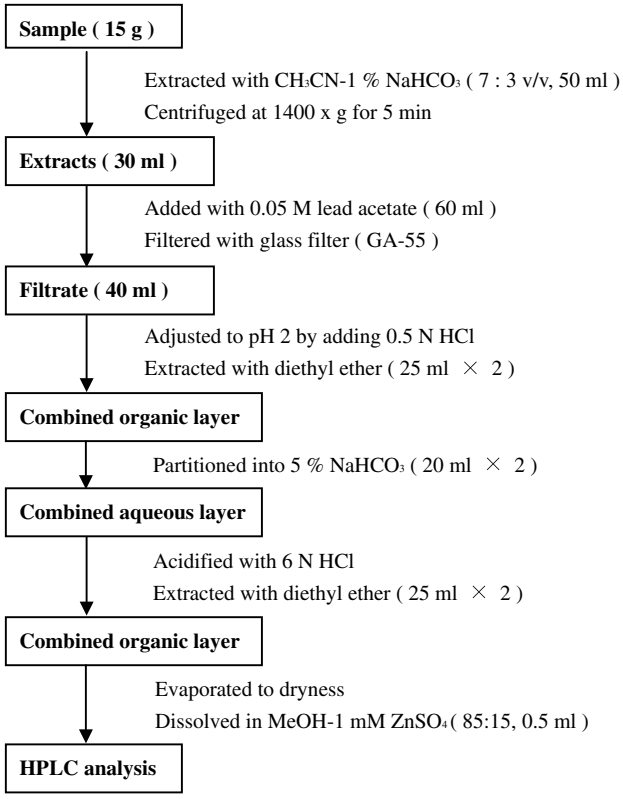

Fig. 2. Schematic procedure for the analysis of CPA.

pH 2 with 6 N HCl and partitioned twice with diethyl ether (25 ml each). Finally, the combined diethyl ether was evaporated in vacuo and dissolved in methanol:1 mM zinc sulfate (85:15, v/v, 0.5 ml). An aliquot (10 μl) of the sample extract was injected into the HPLC column.

2.4. HPLC analysis

The quantification of CPA in corn samples was performed by HPLC with UV detection under the following conditions: a Shimadzu SCL-6A system connected to a Shimadzu SPD-M10AVP photodiode array detector interfaced with a Shimadzu CBM-10A communications bus module and a Shimadzu CLASS-LC10 model FMV-6300 DX 2c computer; reversed phase column – TSK-gel ODS-80TM CTR, 5 μm, 100 × 4.6 mm, (TOSOH Co., Tokyo, Japan); oven temperature 40 °C; wavelength 279 nm; methanol:water (85:15, v/v) as mobile phase A; methanol:4 mM zinc sulfate (85:15, v/v) as mobile phase B; a linear gradient from mobile phase A to mobile phase B in 10 min at a flow rate of 1.2 ml/min. A calibration curve correlating peak-area and concentration was constructed for quantification purposes, using toxin standards. Confirmation of CPA in the sample was achieved using UV and a photodiode array (PDA) detector.

3. Results and discussion

An improved method for the analysis of CPA in corn and rice without the use of toxic solvents such as chloroform was developed. In the present study, the HPLC method reported by Urano et al. (1992) was slightly modified. As expected, CPA formed a strong complex with zinc cations, which eluted in 5 min as a sharp peak. The limit of detection was 0.2 ng for the CPA standard. The response of the UV detector showed a good linearity between 0.5 and 50 ng, as shown in the calibration graph with the linear regression $y = 2003x + 209$ and correlation coefficient $r^2 = 0.9996$. The concentration of CPA in the sample was determined from the calibration graph. Confirmation of the CPA concentration in samples was done using PDA detection.

CPA exhibits an absorption maximum at 279 nm, making the HPLC determination of CPA with UV detection subject to chromatographic interferences from matrix co-extractants. Various types of clean-up and HPLC procedures for CPA in fungal cultures and some agricultural commodities have been studied by several researchers (Goto et al., 1987; Lansden, 1984; Urano et al., 1992). Among them, the method for the determination of CPA developed by Urano et al. (1992), which involves liquid–liquid extraction with chloroform, a silica solid phase clean-up (using chloroform), and UV-HPLC determination with zinc sulfate in methanol gradient elution, has been widely used. However, we observed (Hayashi & Yoshizawa, 2004) difficulty in removing interferences from corn sample extracts using this procedure of Urano et al. (1992). In addition, this method requires the use of chloroform in the clean-up steps. In order to solve these problems, we developed a new method, which combined the HPLC procedure with an extensive liquid–liquid partition clean-up. The clean-up uses pH-dependent extractions and back extractions between organic and aqueous phases. The multiple extraction steps result in clean extracts for corn grits that exhibit no matrix interferences at the same retention time as CPA, as shown in Fig. 3. Until now, chloroform has been widely used as the organic solvent in the partitioning step (Gallagher et al., 1978; Lansden, 1986; Urano et al., 1992) for the analysis of CPA. However, loss of recovery was observed in the back extraction between chloroform and the alkaline solution. Studies showed that diethyl ether was as efficient as chloroform in removing interferences from the sample matrix (data not shown). As a replacement solvent, diethyl ether is considered as a good alternative. Considering the explosive nature of diethyl ether, care should be exercised during its handling.

To evaluate the performance of the method, a recovery test was carried out for corn and rice. The method

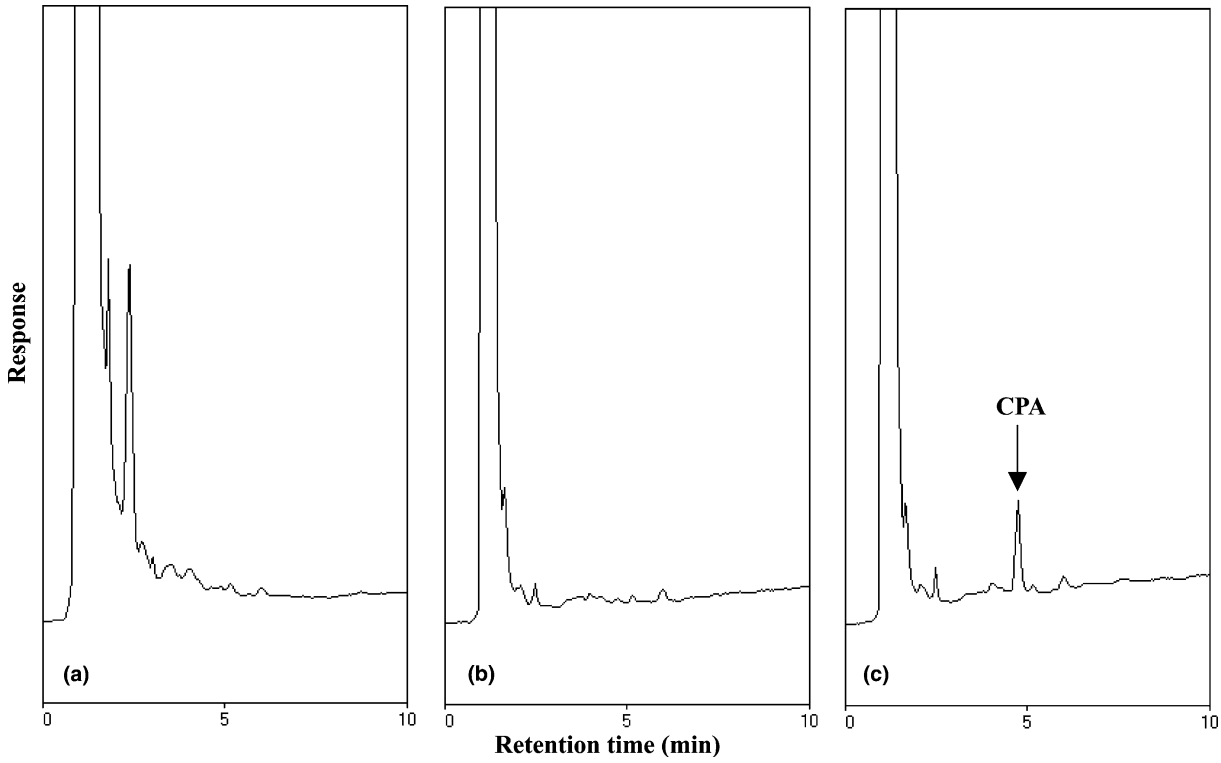

Fig. 3. HPLC chromatograms of (a) unspiked corn grit extract partitioned with diethyl ether, (b) unspiked corn grit extract partitioned with diethyl ether after alkaline back extraction, (c) CPA – spiked (100 ng/g) corn grit extract which was treated as (b). HPLC was performed as described in the text.

Table 1
Recoveries of CPA from spiked corn and rice[a]

Spike level (ng/g)	Corn		Rice	
	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)
25	64.7 ± 0.7	1.1	51.4 ± 1.9	3.7
50	68.5 ± 3.5	5.1	70.4 ± 4.8	6.8
100	74.6 ± 2.8	3.8	82.1 ± 4.5	5.5
200	75.4 ± 1.5	2.0		

[a] All experimental treatments for each sample were performed in triplicate.

described above showed good performance for the determination of CPA in corn and rice samples spiked at different concentrations, as shown in Table 1. The average recoveries from corn spiked with 25, 50, 100, and 200 ng CPA/g, were 64.7%, 68.5%, 74.6% and 75.4%, respectively and, from rice spiked with 25, 50, and 100 ng CPA/g were 51.4%, 70.4%, and 82.1%, respectively. The estimated limit of quantification for corn was 25 ng CPA/g, which was more sensitive than the 50 ng CPA/g reported by Urano et al. (1992).

The method was successfully applied to the CPA analysis of corn samples from the Philippines and rice samples from Thailand. Typical HPLC chromatograms of the samples are shown in Fig. 4. As expected, the method gave a clear chromatogram for CPA extracted from a naturally contaminated corn (sample b). The CPA peak was confirmed using PDA detection, as shown in Fig. 5.

The levels of CPA and AFB_1 in corn and rice samples are summarized in Table 2. AFB_1 was previously analyzed in our laboratory (Arim, 2000; Lipigorngoson et al., 2003). One of the 6 corn samples analyzed was naturally contaminated with CPA at a level of 76 ng/g, and contained AFB1 at a level of 44 ng/g. In the case of Thai rice, which contained AF in a range of 0.6–24 ng/g, CPA was not detected.

Widiastuti et al. (1988) found CPA in 21 of 26 Indonesian corn samples in a range of 30–9220 ng/g. The CPA-positive samples also contained AF (range, 2–1050 ng/g). However, because the quantification of CPA was performed by thin layer chromatography, these data should be interpreted with caution. Apart from this survey, there have been very few reports on the natural occurrence of CPA in agricultural commodities from Southeast Asia. In tropical areas, such as Southeast Asia, members of the genus *Aspergillus* are often the predominant micro-organisms, and toxigenic *A. flavus* isolates are found with high frequency in agricultural commodities (Goto et al., 1999; Lozada, 1995; Shank, Wogan, & Gibson, 1971). Furthermore, human

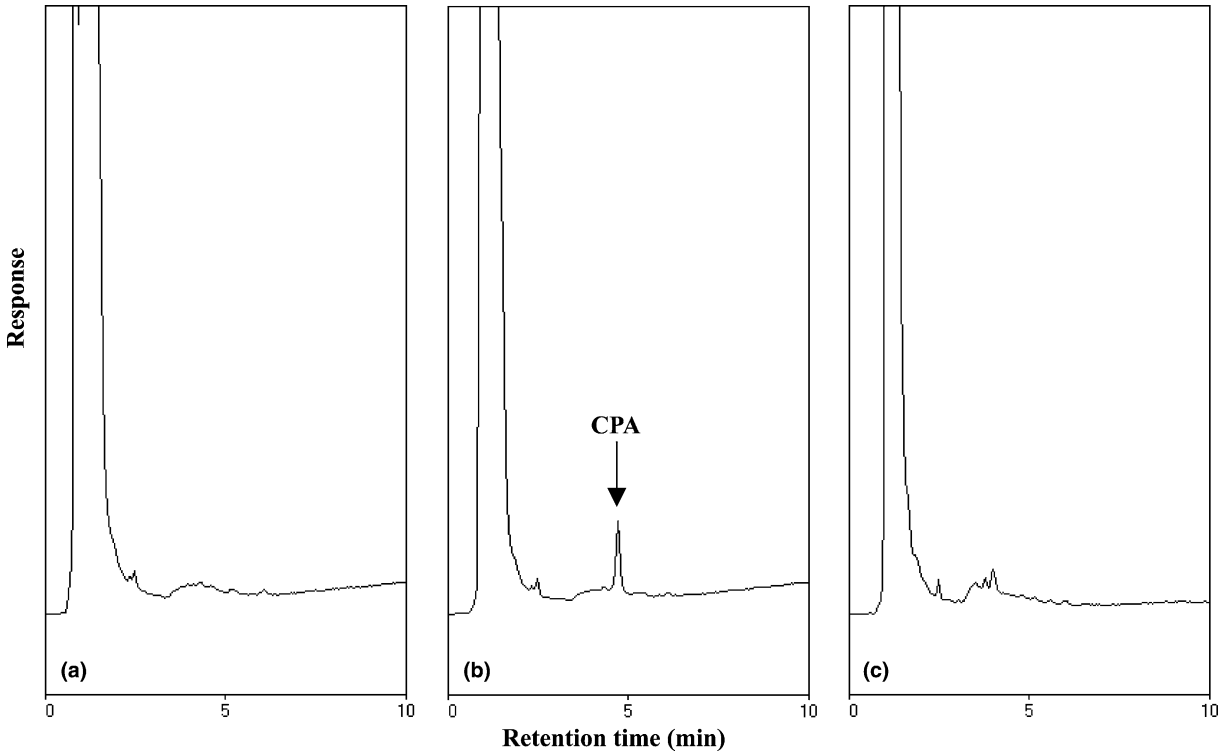

Fig. 4. HPLC chromatograms of (a) uncontaminated Philippine corn sample, (b) Philippine corn sample naturally contaminated with CPA (76 ng/g), (c) uncontaminated Thai brown rice sample. HPLC conditions as in Fig. 3.

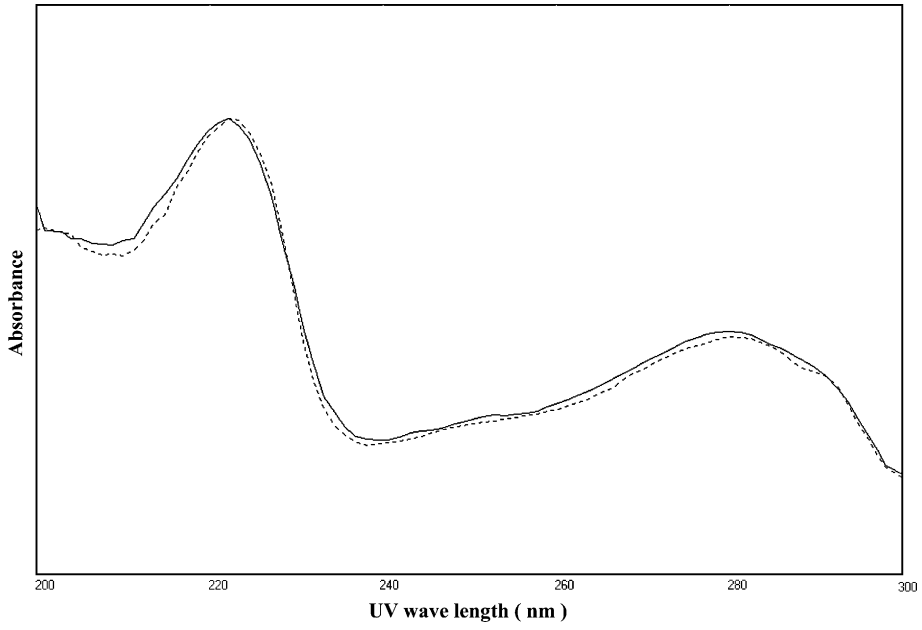

Fig. 5. Confirmation of CPA in naturally contaminated Philippine corn by UV and PDA detection. ———, CPA in contaminated Philippine corn (Fig. 4(b)); - - - - -, CPA standard.

exposure to mycotoxins in staple foods, such as corn and rice, is an important issue in Asia due to the frequency of their intake. Therefore, further surveys for Southeast Asia should be carried out to determine the risk of contamination of CPA and other mycotoxins in agricultural commodities.

Table 2
Natural occurrence of cyclopiazonic acid and aflatoxin B_1 in Philippine corn and Thai rice intended for human consumption[a]

Sample code	Sample type	Origin	Toxins concentration, ng/g	
			Cyclopiazonic acid	Aflatoxin B_1
Philippine corn				
CW 1f	Yellow corn	Ilocos	–[b]	22
CW 1j	Yellow corn	Ilocos	–	29
CW 6d	Yellow com	Iloilo	76	44
CW 6e	Yellow corn	Iloilo	–	2
CW 11e	Yellow corn	South Cotabato	–	1
CW 11f	White corn	South Cotabato	–	1
Thai rice				
PR-WH-27	Polished rice	Chiang Mai	–	24.0
PR-WH-26	Polished rice	Chiang Mai	–	13.5
PR-SK-4	Polished rice	Chiang Mai	–	2.6
BR-SS-15	Brown rice	Chiang Mai	–	2.8
BR-SS-10	Brown rice	Chiang Mai	–	2.4
BR-SS-17	Brown rice	Chiang Mai	–	2.8
BR-SS-9	Brown rice	Chiang Mai	–	1.2
PR-SS-24	Polished rice	Chiang Mai	–	1.0
BR-SS-11	Brown rice	Chiang Mai	–	0.8
PR-SS-22	Polished rice	Chiang Mai	–	0.6

[a] Data on aflatoxin B_1 contamination are cited from the publications: Philippine corn (Arim, 2000) and Thai rice (Lipigorngoson et al., 2003).
[b] Not detected (below the quantification limit, 25 ng/g).

4. Conclusion

The improved method for detection of CPA described in the present study does not use chloroform and employs clean-up procedures that involve extraction with acetonitrile–alkaline water, precipitation of protein with lead acetate, liquid partitioning with diethyl ether and determination by HPLC with UV and PDA detection. The method was successfully applied to the analysis of CPA in corn and rice samples from the Philippines and Thailand. The natural occurrence of CPA in corn from the Philippines was detected, but not in rice from Thailand. This method has potential application in the determination of CPA in other foods. Further surveys of CPA contamination in corn and rice from Southeast Asian countries will be continued.

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