

Immunochemical Method for Cyclopiazonic Acid

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1. Introduction

Cyclopiazonic acid (CPA) (Fig. 1) is a toxic, indole tetramic acid that was originally isolated from *Penicillium cyclopium* Westling (1) and subsequently reported to be produced by numerous species of *Penicillium* and *Aspergillus* (2). Among the species of *Aspergillus* that produce CPA is *A. flavus*, which is primarily known as a producer of the aflatoxins and is a frequent contaminant of corn, peanuts, and other commodities. The taxonomy of *Penicillium* species that produce CPA has undergone several revisions, but Pitt et al. (3) concluded that the correct name for most saprophytic Penicillia that produce CPA is *P. commune* with *P. palitans* as a synonym. This would include the original isolate variously identified as *P. cyclopium* (1), *P. griseofulvum* (4), and *P. verrucosum* var. *cyclopium* (5). Pitt et al. (3) also classified all molds used in the manufacture of white cheeses that produce CPA as *P. camembertii*. Based primarily on chemotaxonomical features coupled with conidial colors on Czapek yeast autolysate agar, Lund (6) concluded that *P. palitans* was not just synonymous with *P. commune*, but was actually a distinct species. Despite some confusion with regard to the taxonomy of CPA-producing Penicillia, the fact remains that the various species of *Penicillium* and *Aspergillus* that produce CPA are ubiquitous and abundant in nature and are common contaminants of commodities that go into foods and feeds. Therefore, the potential for the contamination of commodities with CPA is widespread (7).

Natural occurrence of CPA has been reported in corn (8-10), peanuts (10,11), cheese (12,13), millet (14), sunflower (15), and various feeds and feedstuffs (16). The toxin has also been shown to accumulate in meat and eggs of chickens (17,18) and the milk of sheep (18) dosed with CPA.

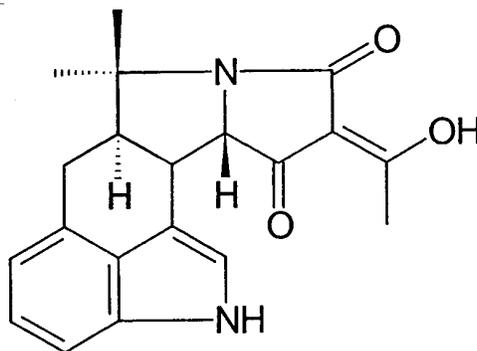


Fig. 1. Chemical structure of cyclopiazonic acid.

Toxicosis resulting from consumption of CPA-contaminated food or feed has not been proven unequivocally, but CPA has been strongly implicated as the causative agent or one of the causative agents in several mycotoxicoses. Whereas the aflatoxins were certainly largely responsible for the outbreak termed turkey "X" disease (19), Cole (20) later presented a case of strong circumstantial evidence for the involvement of CPA. This case was strengthened when CPA was detected at a concentration of 31 $\mu\text{g}/\text{kg}$ in a sample of groundnut cake that had been saved from the original turkey "X" disease (21). CPA was strongly implicated in a case of "kodu poisoning" in man (14). The kodu millet that produced symptoms of giddiness and nausea in two separate instances contained CPA and was heavily infected with CPA-producing strains of *A. flavus* and *A. tamarii*. CPA was also considered to be responsible for the death of quails that consumed feed containing 6000 $\mu\text{g}/\text{kg}$ of the toxin (22).

Many types of methods have been used to detect and quantify CPA in fungal cultures and various agricultural commodities, with thin-layer chromatography (TLC) being the most popular. TLC is typically performed on silica gel plates that have been pretreated with oxalic acid to prevent tailing of CPA (23). In addition, modification of certain solvent systems with ammonia or acetic acid have been used to prevent tailing (24,25). CPA can be visualized on TLC plates as a blue-violet spot after spraying with Ehrlich's reagent (11). TLC has been used to quantify CPA in cheese (26), corn (8,9,27), peanuts (11,21), sunflower (15), millet (14), and milk and eggs (18).

High performance liquid chromatographic (HPLC) methods have also been used to determine CPA in various matrices. Lansden (28) reported a reversed-phase system for peanuts that used a C_8 or C_{18} column and a mobile phase containing 4-dodecyldiethylenetriamine, zinc acetate, ammonium acetate, 2-propanol, and acetonitrile with UV detection at 284 nm. The detection limit

for pure CPA was 4 ng and recoveries of CPA from spiked peanuts ranged from 72.9% to 85.9%. Goto et al. (29) used normal-phase HPLC with a silica gel column, mobile phase consisting of ethyl acetate-2-propanol-25% aqueous ammonia (55:20:5, v/v/v), and a SPD-6A spectrophotometer at 284 nm to achieve a detection limit for pure CPA of 0.2 ng. Using an extraction solvent of chloroform-85% phosphoric acid and a silica cleanup cartridge, they reported an 82% recovery of CPA from maize with a lower detection limit of 0.1 µg/g. Urano et al. (30) described a reversed-phase method using a C₁₈ column and a linear gradient of 0–4 mM ZnSO₄ in methanol-water (85:15, v/v) to quantify CPA in corn and peanuts, with quantitation limits of about 50 and 100 ng/g, respectively. The method was used in a 1990 survey of corn and peanuts which showed extensive contamination of both crops with CPA and aflatoxins (10). Matsudo and Sasaki (31) reported a simple HPLC method for analyzing extracts of fungal cultures. The system consisted of a C₁₈ column with a mobile phase of 50 mM H₃PO₄ plus 1 mM ZnSO₄-acetonitrile (45:55) and UV detection at 284 nm. Indomethacin was added to sample extracts as an internal standard, and CPA concentrations were calculated on the basis of the ratio of the peak area of CPA to that of the internal standard. The detection limit for CPA was 0.3 ng. Sobolev et al. (32) recently reported a normal phase ion-pair partition HPLC system that was used to detect CPA simultaneously with other metabolites of various *Aspergillus* species. A silica gel column with a mobile phase of n-heptane-2-propanol-n-butanol-water-tetrabutylammonium hydroxide (2560 + 900 + 230 + 32 + 8, v/v) and a diode array detector allowed separation and detection of at least seven metabolites, including CPA, with a detection limit for CPA of 5 ng/injection.

Several spectrophotometric methods have been used for CPA determination. Rathinavelu and Shanmugasundaram (33) used TLC to purify extracts, eluted CPA from TLC plates, and added p-dimethylaminobenzaldehyde plus HCl to develop color which was measured with a colorimeter at 560 nm. Variations and improvements to this basic method have been reported by Rao and Husain (34), Chang-Yen and Bidasee (35), and Šimůnek et al. (36), and a detection limit in corn and poultry feed of 80 µg/kg was reported (35).

The application of capillary electrophoresis to analysis of CPA in milk has been reported recently (37). The micellar electrokinetic capillary chromatography method involved extraction of milk with basic methanol-water followed by partitioning and Sep-Pak cleanup. The analytical response was linear from 40 ppb to 100 ppm of CPA in milk and recoveries were 78–81% over the range of 20–500 ppb. The minimum quantifiable concentration of CPA in spiked milk samples was 20 ppb.

Enzyme-linked immunosorbent assays (ELISA) have been developed and applied successfully to the analysis of CPA in fungal cultures and cheese (38–41).

However, it was found that when agricultural commodities were analyzed with an immunoassay, interferences from the sample matrix produced losses in the sensitivity usually associated with these assays (42). Therefore, an immunoaffinity column containing a monoclonal antibody with high affinity to CPA was developed for the cleanup of sample extracts before ELISA analysis (43). Use of the immunoaffinity column prior to ELISA analysis improved detection limits for CPA in corn, mixed feed, and peanuts from 100, 300, and 600 ng/g, respectively, to 2.0, 4.4, and 4.7 ng/g, respectively. It was also shown that the immunoaffinity column could be regenerated for reuse at least 10 times by washing with equilibrating buffer and storing in a cold room overnight.

Most published analytical methods for the determination of CPA require extensive, time-consuming cleanup procedures to achieve accurate quantitation by one of the previously described techniques. In many cases, extraction is followed by several liquid-liquid partitioning steps and further cleanup by column, cartridge, or TLC. The purpose of this work was to apply simple immunoaffinity column cleanup to peanut extracts prior to analysis by HPLC.

2. Materials

1. Vertical cutter mixer (VCM) or food processor (e.g. Robot Coupe RSI6Y-1 or Cuisinart DLC-8S).
2. Blender: Waring with 500 mL glass jar.
3. Filter papers: 15 cm coarse fluted filter paper (P8, Fisher Scientific, Atlanta, GA); 4.25 cm glass microfiber filters (GF/C Whatman Ltd., Maidstone, England).
4. Test tubes: borosilicate glass culture tubes with plain end (20 × 150 mm).
5. Flat-bottom boiling flask (1 L).
6. Rotary evaporator (Büchi Rotavapor).
7. CPA-specific immunoaffinity columns (Prepare columns as previously described (43) by coupling a CPA-specific monoclonal antibody to Sepharose gel and loading a filter tube with 0.2 mL of CPA immunogel in 0.57 mL of slurry. Fill columns with phosphate buffered saline [PBS] containing 0.02% sodium azide and store at 4°C.).
8. Heating block with 9 ports for 4 mL vials.
9. High purity compressed nitrogen.
10. Vortex mixer: Touch-Mixer (Fisher).
11. Vial: 4 mL borosilicate clear autosampler vial with screw cap and PTFE septa (National Scientific, Lawrenceville, GA).
12. HPLC System.
 - a. Pump: Model 515 (Waters Chromatography, Milford, MA).
 - b. Injector: Model 7125 syringe loading sample injector with 20 µL loop (Rheodyne, Cotati, CA) or Model 712 WISP autosampler (Waters).
 - c. Detector: Model SPD-10A diode array with Class-VP chromatography data system (Shimadzu, Kyoto, Japan) or Model 490 E programmable multi wavelength UV detector at 282 nm (Waters).

- d. Column: Zorbax Rx-SIL 250 × 4.6 mm id, packed with 5 μ M silica gel (MAC-MOD Analytical).
13. Solvents for HPLC: HPLC grade hexane and reagent alcohol (Fisher).
14. Water: Distilled, purified with Milli-Q Water System (Millipore, Burlington, MA).
15. TRIS buffer: 1 g TRIS in 10 mL water.
16. Phosphate buffered saline (PBS): 10 mM, pH 7.4 (Sigma, St. Louis, MO).
17. Extraction solvent: 70:30 methanol/1% sodium bicarbonate in water.
18. HPLC Mobile Phase: 500:275:16 hexane/reagent alcohol/TRIS.
19. HPLC Standard: Stock solution: Dissolve 1 mg of CPA in 10 mL of ethyl acetate plus 1% acetic acid; Working standard: Evaporate 20 μ L of stock solution to dryness and redissolve in 2 mL of HPLC mobile phase (final concentration of 1 ng/ μ L).

3. Methods

3.1. Sample Preparation and Extraction

1. Grind peanuts in VCM or food processor to a homogeneous paste (about 6 min).
2. Transfer a 50 g subsample to a Waring blender, add 150 mL of extraction solvent, and blend at high speed for 2 min.
3. Filter extract through fluted filter paper, transfer 15 mL to a test tube, add 2 mL of a saturated solution of sodium bicarbonate, and place in a freezer for at least 30 min.
4. Suction filter contents of test tube through microfiber filter paper into 100 mL boiling flask.
5. Evaporate solvent using a rotary evaporator at 50°C until the volume in the flask is about 4–6 mL.

3.2. Immunoaffinity Column Chromatography

1. Wash column with 10 mL of PBS to remove sodium azide and apply extract from evaporating flask.
2. Rinse flask three times with 2 mL of 1% sodium bicarbonate and add to affinity column.
3. Load column at a flow rate of 1 mL/min and wash with 15 mL of purified water.
4. Wash column with 2 mL of 70:30 water/methanol.
5. Elute CPA with 2 mL of methanol at a flow rate of 0.5 mL/min into vial.
6. Evaporate solvent under stream of nitrogen in heating block.
7. Redissolve eluate in 500 μ L of HPLC mobile phase and mix well with vortex mixer.

3.3. Liquid Chromatography

1. Set mobile phase flow rate at 1.2 mL/min or adjust to achieve desired retention time of CPA. Let system equilibrate until steady baseline is achieved.
 2. Inject 20 μ L of HPLC working standard (contains 20 ng of CPA and is equivalent to 100 ng/g in peanuts) and calibrate data system at 100 ng/g for external standard quantitation, which corresponds to 100% recovery of CPA from peanuts.
 3. Inject 20 μ L of prepared samples.
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Table 1
Method Performance for LC Determination of CPA
with Immunoaffinity Column Cleanup

	CPA Added (ng/g) ^a	Recovery (%)	RSDr (%) ^b
Peanuts ^c	10	90.8	
	100	83.7	
Peanut Extract ^d	100	93.8	

^aFive determinations were made at each spike level.

^bRSDr = within laboratory coefficient of variation.

^c50 g of CPA-free ground peanuts. Limit of detection of the method = 2.5 ng/g.

^d15 mL of filtered CPA-free peanut extract.

3.4. Method Performance

The recovery of CPA from spiked peanuts and peanut extract and the repeatability of the method as measured by the relative standard deviation (RSDr) are presented in **Table 1**. The recovery from spiked peanuts ranged from 83.7–90.8%. The improvement in recovery to 93.8% and the low RSDr associated with spiked peanut extract indicated that the immunoaffinity column cleanup associated with the HPLC system provided excellent recovery of CPA with highly repeatable results.

4. Notes

1. Take safety precautions. Wear protective clothing, gloves, and eye protection. See the Material Safety Data Sheets or equivalent for each reagent. Dispose of waste solvents according to applicable environmental rules and regulations.
2. Addition of saturated sodium bicarbonate to the initial filtrate (*see Subheading 3.1.3.*) and freezing causes precipitation of impurities that interfere with complete binding of CPA by the immunoaffinity column. This improved recovery from 55–60% to that shown in **Table 1**.
3. LC analysis of CPA standards was linear ($r = 0.9999$) over the range of 1–1000 ng of CPA per injection (5–5000 ng/g). However, because the binding capacity of the immunoaffinity column is 4 μ g, the upper limit for quantitation is 800 ng/g. For test portions that measure higher, another subsample must be extracted and subjected to the cleanup procedure and LC analysis.
4. The effectiveness of the immunoaffinity column is illustrated by comparing **Fig. 2** (chromatogram of 50 g of peanuts spiked with 100 ng/g of CPA and cleaned up with the immunoaffinity column) with **Fig. 3** (chromatogram of 50 g of peanuts spiked with 100 ng/g of CPA and cleaned up using liquid-liquid partition and solid phase extraction).
5. Use of a diode array detector enables confirmation of the identity of CPA in a sample by comparison of its UV spectrum with that of authentic CPA.

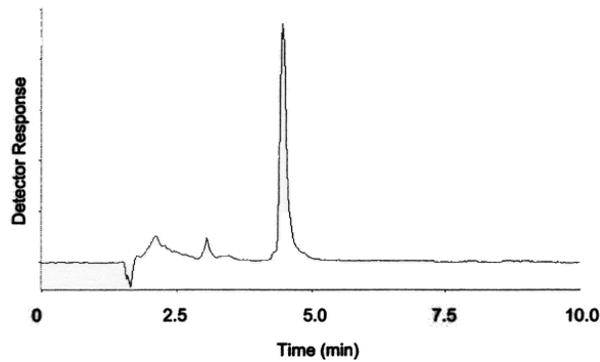


Fig. 2. Chromatogram of an extract of peanuts spiked with 100 ng/g of CPA and cleaned up using immunoaffinity column chromatography.

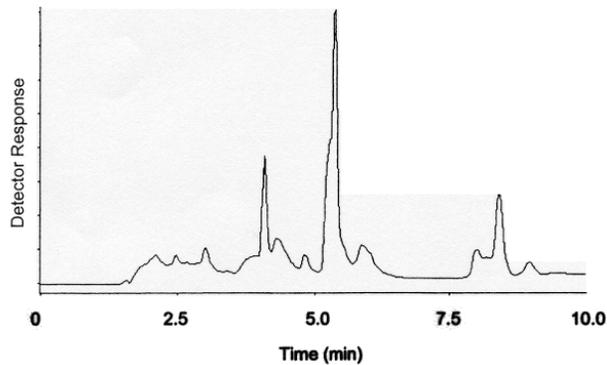


Fig. 3. Chromatogram of an extract of peanuts spiked with 100 ng/g of CPA and cleaned up with liquid-liquid solvent partition followed by solid phase extraction (30). CPA co-eluted with a major interfering compound at a retention time of 5.4 min. The HPLC system was not optimized for this cleanup procedure.

6. The method was used to analyze peanuts grown under late-season drought stress, conditions that favor contamination of peanuts with CPA and aflatoxins. Results of analyses for CPA using the immunochemical method along with results of analyses for aflatoxins in different seed size categories are shown in Table 2.

Table 2
Concentrations of CPA and Aflatoxin in Peanuts Grown Under Late-season Drought Stress

Peanut Seed Size	Weight (g) ^a	CPA (ng/g) ^b	Aflatoxin (ng/g) ^{a,b}
Jumbo		3.0	26.5
Medium		44.9	0.0
Number 1		145.6	122.4
Oil Stock		170.5	0.6
Damage ^c		8105.0	7094.3

^aValues are the means of three replicated, drought-stressed plots (4.0 × 5.5 m).

^bValues are the total of aflatoxins B₁, B₂, G₁, and G₂.

^cDamaged seed were hand-picked from each seed size category and combined for one analysis per plot.

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