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Occurrence of Cyclopiazonic Acid in Peanuts

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Samples of segregation 3 farmer stock peanuts from the 1980 southeastern United States growing season were analyzed for the presence of cyclopiazonic acid and aflatoxins. Cyclopiazonic acid appeared in 21 of 27 loose-shell kernel fractions at a range of 32 to 6,525 μg/kg and in 4 of 21 sound mature kernel fractions at a range of 32 to 130 μg/kg. Aflatoxins were detected in 26 of 27 loose-shell kernel fractions and in 20 of 21 sound mature kernel fractions. Cyclopiazonic acid used at 105 and 210 μg/kg to spike peanut samples was recovered at an average rate of 93.3%, with ranges of 89 to 119 and 166 to 221 μg/kg, respectively. The minimum detection limit on oxalic acid-impregnated silica gel plates was 26 ng.

Cyclopiazonic acid (CPA), a toxic indole tetramic acid, was first isolated from *Penicillium cyclopium* Westling (5). It was subsequently reported to be a metabolite of *Aspergillus flavus* Link (6). Gallagher et al. (3) have reported that 28 of 54 *A. flavus* isolates studied produce CPA, whereas 18 produce aflatoxins. However, 40 isolates of *Aspergillus parasiticus*, also associated with aflatoxin contamination, have been found not to produce CPA (J. W. Dorner, M.S. thesis, Auburn University, Auburn, Ala. 1982).

Currently in the United States, farmer stock peanuts are checked for visible *A. flavus* during the grading process at buying locations. Peanut lots found to be contaminated are labeled as segregation 3 peanuts and converted to oil stock and nonedible products. Since the grading process does not guarantee 100% removal of contamination, chemical methods are used to detect aflatoxins in segregation 1 peanuts during the processing of edible nuts and nut products (1). No other mycotoxins are currently assayed by quality control regimes in the marketing of edible peanuts and peanut products.

The purpose of this study was to determine if CPA is a natural contaminant in peanuts.

MATERIALS AND METHODS

Samples of segregation 3 farmer stock peanuts were collected from multiple southeastern United States growing locations during the peanut grading process at several buying points during the 1980 crop year. A 34-kg sample lot was taken from each segregation 3 farmer stock lot and separated into a loose-shell kernel (LSK) fraction and a sound mature kernel (SMK) fraction by standard shelling practices. Each fraction was ground in a Dickens Hammermill (Federal-State Inspection Service, Albany, Ga.) and thoroughly mixed before removal of a 1,100-g sample for aflatoxin analysis by the CB method (1). The bulk of each fraction was stored at 0°C before analysis for CPA. The complete range of samples analyzed for both aflatoxins and CPA were as follows: 13 samples having both LSK and SMK fractions, 14 samples having only LSK fractions, and 8 samples having only SMK fractions. A set of six Federal-State Inspection Service grade check samples for 1981, classified as segregation 1, were processed in a similar manner to obtain both LSK and SMK fractions.

Reference thin-layer chromatography (TLC) spotting solutions were made by dilution of a stock solution containing 1.909 mg of CPA per ml in methanol. Oxalic acid-impregnated silica (Ox-Si) gel plates were made by dipping commercial silica gel 60 F-254 plates (5 by 10 cm; catalog no. 5760; E. Merck AG, Darmstadt, Germany) into freshly prepared 0.1 M oxalic acid in methanol. The plates were then air dried and reactivated at 120°C for 1 h and stored over anhydrous calcium sulfate. Oxalic acid solutions stored for more than 1 day did not produce equivalent plates. Preparative TLC plates were prepared from Kieselgel G-HR silica gel (Macherey-Nagel & Co., GmbH, Duren, West Germany) according to the directions of the manufacturer except that 0.2 M oxalic acid was substituted for water. Ehrlich spray reagent was made by dissolving 1 g of *p*-dimethylaminobenzaldehyde in 75 ml of 95% ethanol and adding 25 ml of concentrated hydrochloric acid.

The method of analysis used in this study is similar to that used by Gallagher et al. (3) for analysis of CPA in corn. Samples of peanut meal were de-fatted with petroleum ether and air dried before analysis. A 100-g sample was weighed and blended with 250 ml of 20% methanol in chloroform at low speed until most of the liquid was absorbed. An additional 250 ml of 20% methanol in chloroform and 2 ml of 85% phosphoric acid were added, and the contents were blended for 3 min. The extract was vacuum filtered on glass fiber filter paper. A 100-ml portion was flash evaporated at 40°C to one-half volume. The extract was then transferred to a centrifuge separatory funnel and extracted

TABLE 1. Aflatoxin and CPA contamination of segregation 3 peanuts (1980 crop)

Sample	LSK		SMK	
	Aflatoxin (μg/kg)	CPA (μg/kg)	Aflatoxin (μg/kg)	CPA (μg/kg)
A	0	327	16	0
B	43	0	3	0
C	64	0	3	0
D	64	130	0	0
E	78	0	3	0
F	376	520	291	65
G	425	981	128	39
H	465	0	6	0
I	535	0	182	0
J	1,268	520	71	0
K	1,795	653	56	0
L	2,348	1,044	52	32
M	3,495	1,088	550	0

with 50 ml of 0.5 M $NaHCO_3$ by vigorous shaking and centrifuging for 10 min at 150 × *g*. The upper layer was washed with an additional 50 ml of chloroform and recentrifuged. The upper layer was then acidified by the careful addition of 4.5 ml of 6 N HCl. After effervescence ceased, 50 ml of dichloromethane was added to the funnel, which was vigorously shaken and then centrifuged. The lower layer was drained into a 100-ml pear-shaped flask and flash evaporated at 40°C to dryness. The residue was reconstituted in 250 μl of acetonitrile. For initial screening of the extracts, each Ox-Si plate was spotted with 10 μl of sample and 2 or 3 μl of CPA standard. The plates were developed in methyl isobutyl ketone-chloroform (1:4), and CPA was detected by spraying with Ehrlich reagent and gently heating.

Quantitation of CPA-positive fractions was accomplished by spotting 2, 3, or 5 μl of a sample and a sequential range of 2 to 5 μl of CPA standards on Ox-Si plates. The amount of sample to be used for quantitation was determined by using the survey results as a relative guide. When the screening procedure indicated only trace amounts, 15 μl of sample was used. The quantitative plates were developed in freshly prepared developing solvent, and sample was detected with freshly prepared Ehrlich reagent to minimize variations among plates. Calculations were performed with the following formula: $\mu g/kg = (S \cdot Y \cdot V)/(X \cdot W)$, where S is the unknown microliter amount of CPA standard, Y is the concentration of CPA standard (micrograms per milliliter), V is the microliter amount of sample extract after the final dilution, X is the microliter amount of sample extract with intensity equal to X (CPA standard), and W is the gram amount of sample in 100 ml of initial extract.

The recovery rate for the extraction routine was determined by spiking four 100-g replicate portions of de-fatted peanuts with aflatoxins at levels equivalent to 105 and 210 μg/kg.

The isolation of naturally occurring CPA from one of the most highly contaminated LSK fractions was performed by extracting 600 g of de-fatted sample with acidified 20% methanol–chloroform. The extract was partitioned among 1.5 liters of hexane, 2 liters of methanol, and 100 ml of water. The upper phase was reduced in volume and partitioned between 300 ml of chloroform and 0.5 M $NaHCO_3$. After separation of the phases, the upper aqueous phase was washed with chloroform, acidified with hydrochloric acid, and extracted twice with dichloromethane. The dichloromethane extracts were combined, reduced to an oily residue by vacuum evaporation, and dissolved in acetonitrile. Crude CPA was obtained by streaking three Ox-Si preparative TLC plates with acetonitrile extract. The appropriate CPA bands were scraped from the plates, and the CPA was eluted from the Ox-Si gel with 30 ml of 50% chloroform–methanol. Oxalic acid and minor impurities were removed by partitioning the CPA into 10 ml of 0.5 M $NaHCO_3$, acidifying with hydrochloric acid, and repartitioning into 5 ml of dichloromethane. The dichloromethane was evaporated to ca. 50 μl under a nitrogen stream before infrared analysis.

RESULTS

The results of the analysis for aflatoxin and CPA contamination of peanuts are shown in Table 1. In sample lots in which both LSK and SMK fractions were present, the incidences of aflatoxin contamination were equivalent. However, absolute values of aflatoxin concentrations in the LSK fractions were generally much higher than absolute values for the SMK fractions. These results closely parallel data published by Dickens et al. (2) on drought-stressed segregation 3 peanuts for the 1968 crop year in North Carolina and data obtained for drought-stressed peanuts in specially constructed environmental plots (4).

The incidence of CPA contamination differed markedly from that of aflatoxin contamination. In the sampled lots shown in Table 1, only 3 of 13 SMK fractions had detectable amounts of CPA, whereas 8 of 13 LSK fractions were contaminated with CPA. These results were further corroborated by analyses of sample lots in which only LSK or SMK fractions were present (Table 2). In these lots, 13 of 14 LSK fractions contained CPA, whereas 1 of 8 SMK fractions were contaminated. As was the case for aflatoxin contamination, CPA concentrations in the SMK fractions were substantially less than concentrations in the LSK fractions. The overall incidence of CPA in segregation 3 peanuts was 78% for LSK fractions but only 19% for SMK fractions.

Analyses of the segregation 1 check samples found three LSK fractions contaminated at 15 to 25 μg of aflatoxin per kg and a separate LSK fraction with a trace of CPA. No SMK fractions were found to be contaminated, and two samples were judged to be free of contamination in both LSK and SMK fractions.

Recovery studies on de-fatted peanuts spiked at levels equivalent to 105 and 210 μg/kg showed an average recovery of 93.3% for four replications at each level, with ranges of 89 to 119 and

TABLE 2. Aflatoxin and CPA in peanuts

Type of sample(s)	LSK				SMK			
	Aflatoxin		CPA		Aflatoxin		CPA	
	No. positive	Range (μg/kg)	No. positive	Range (μg/kg)	No. positive	Range (μg/kg)	No. positive	Range (μg/kg)
LSK and SMK	12 (13)[a]	43–3,495	8 (13)	130–1,088	12 (13)	3–550	3 (13)	32–65
LSK only	14 (14)	2–5,091	13 (14)	32–6,525	—[b]		—	
SMK only	—		—		8 (8)	22–194	1 (8)	130
Segregation 1[c]	3 (6)	15–25	1 (6)	Trace	0[d] (6)		0[d] (6)	

[a] Numbers in parentheses indicate total numbers of analyses.
[b] —, No sample.
[c] Federal-State Inspection Service grade check samples.
[d] None detected at 1 μg/kg for aflatoxin and 32 μg/kg for CPA.

166 to 221 μg/kg, respectively. The minimum detection TLC limit, determined with sequentially smaller amounts of a standard solution of CPA, was 26 ng. This limit gave a calculated limit of detection of 32 μg/kg for the initial screening procedure.

A small quantity of CPA was isolated from a natural occurrence of highly contaminated peanuts to confirm the presence of the mycotoxin. The CPA isolate had TLC R_f values and color reactions equivalent to those of authentic CPA. The infrared spectrum of the natural CPA isolate matched that of authentic CPA, providing that the authentic material was also pretreated by dissolution in bicarbonate, acidification with HCl, and partitioning into dichloromethane before infrared analysis.

DISCUSSION

Prolonged drought stress during the latter part of the peanut growing season has been associated with a high incidence of *A. flavus* invasion and concomitant aflatoxin contamination (2). The fact that CPA is also a possible metabolite of *A. flavus* has already been established (6). The results of our study confirm the possibility that CPA is a natural contaminant of peanuts. Table 2 indicates that CPA contamination may occur at fairly high levels in specific samples. Table 1 shows that CPA contamination may occur without any detectable aflatoxin formation. However, it is apparent that the frequency of CPA contamination appears to be lower than that of alfatoxin contamination (Tables 1 and 2). This is especially true for the segregation 3 SMK fractions and the segregation 1 check samples. Whether this lower frequency of CPA contamination is a general condition of all growing areas and varieties of peanuts has yet to be established.

The absolute values of aflatoxin and CPA concentrations listed in Tables 1 and 2 cannot be directly compared because it was necessary to de-fat the samples before extraction for CPA analysis. Preliminary experiments with full-fat samples were plagued with emulsions which formed during the liquid-liquid extractions and which could not be completely resolved, even by prolonged centrifugation. Recovery experiments performed by spiking the chloroform extracts of full-fat peanuts typically resulted in an average recovery of less than 80%, indicating that the emulsions were detrimental to maximum CPA transfer between phases in the liquid-liquid extractions. Since the primary purpose of this report was to ascertain the frequency of CPA contamination of peanuts and not to provide an absolute, quantitative procedure, a sacrifice in absolute quantitation was made to ensure more consistent detection of CPA contamination at low levels.

Although the peanuts used in this study would not have entered the food market, the results indicate that CPA is a potential contaminant of edible peanuts. The economic importance of and health risks involved in CPA contamination of peanuts and other agricultural commodities has not been fully investigated, especially in regard to possible synergistic effects with other naturally occurring mycotoxins.

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LITERATURE CITED

1. **Association of Official Analytical Chemists.** 1980. Official methods of analysis, 13th ed., p. 418–419. Association of Official Analytical Chemists, Washington, D.C.
2. **Dickens, J. W., J. B. Satterwhite, and R. E. Sneed.** 1973. Aflatoxin-contaminated peanuts produced on North Carolina farms in 1968. J. Am. Peanut Res. Educ. Assoc. Inc. 5:48–58.
3. **Gallagher, R. T., J. L. Richard, H. M. Stahr, and R. J. Cole.** 1978. Cyclopiazonic acid production by aflatoxigenic and non-aflatoxigenic strains of *Aspergillus flavus*. Mycopathologia 66:31–36.
4. **Hill, R. A., P. D. Blankenship, R. J. Cole, and T. H. Sand-**

ers. 1983. Effects of soil moisture and temperature on preharvest invasion of peanuts by the *Aspergillus flavus* group and subsequent aflatoxin development. Appl. Environ. Microbiol. 45:628–633.
5. **Holzapfel, C. W.** 1968. The isolation and structure of cyclopiazonic acid, a toxic metabolite of *Penicillium cyclopium* Westling. Tetrahedron 24:2101–2119.
6. **Luk, K. C., B. Kobbe, and J. M. Townsend.** 1977. Production of cyclopiazonic acid by *Aspergillus flavus* Link. Appl. Environ. Microbiol. 33:211–212.