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Natural co-occurrence of aflatoxin and cyclopiazonic acid in peanuts grown in Argentina

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Natural occurrence of aflatoxins and cyclopiazonic acid (CPA) contamination in peanuts was investigated. Co-occurrence of CPA and aflatoxins was detected in two of 50 samples analysed. The levels of these toxins found in positive samples were 4300 and 493 $\mu g\,kg^{-1}$ for CPA, 625 and 435 $\mu g\,kg^{-1}$ for aflatoxin B_1 (AFB_1), and 625 and 83 $\mu g\,kg^{-1}$ for aflatoxin G_1 (AFG_1), respectively. Levels of CPA contamination in the positive samples were similar to those registered in other substrates. This is the first report of natural co-occurrence of CPA and aflatoxins in Argentina.

Keywords: cyclopiazonic acid, aflatoxins, peanuts, co-occurrence

Introduction

Cyclopiazonic acid (CPA) is a toxic secondary metabolite produced by several species of *Penicillium* and *Aspergillus*. These fungi are widely distributed in nature. CPA has been found as a natural contaminant of corn (Gallagher *et al.* 1978), peanuts (Lansden and Davidson 1983), cheese (LeBars 1979) and millet (Rao and Husain 1985). Chemically known as indole tetramic acid, CPA is toxic to many animal species, e.g. pig, chicken and dog. Observed clinical signals were weight loss, diarrhoea, depression, opisthotonus, convulsions and death (Voss 1990). CPA could also be accumulated in animal tissue or transmitted to milk and eggs (Norred *et al.* 1988, Dorner *et al.* 1994).

Since the original report of CPA production by *Aspergillus flavus* (Luk *et al.* 1977), the possibility of synergistic effects of CPA and aflatoxins co-occurring in foods has been considered, although most effects are additive (Smith *et al.* 1992). CPA seems to be a more common metabolite of *A. flavus* than aflatoxins (Trucksess *et al.* 1987) and thus is also likely to be present in aflatoxin-contaminated foods.

Strains of *A. flavus* capable of producing high levels of CPA are frequently isolated from crops in Argentina. Resnik *et al.* (1996a) found that all but one of 34 strains of *A. flavus* isolated from corn produced CPA on this substrate at levels ranging from 833 to 10 000 $\mu g\,kg^{-1}$, while only five strains could produce AFB_1 between 29 and 115 $\mu g\,kg^{-1}$. Vaamonde *et al.* (1996) reported that 93% of 45 *A. flavus* strains (30 strains isolated from peanuts, 15 from wheat) produced CPA and that 60% of isolates from peanuts produced CPA and aflatoxins simultaneously.

The natural occurrence of aflatoxins in Argentinian corn and peanuts has been detected (Varsavsky *et al.* 1985, Resnik *et al.* 1996b) but there are no surveys on CPA occurrence. Taking into account the high frequency of isolation of *A. flavus* strains able to produce aflatoxins and/or CPA, the purpose of present work was to investigate the natural co-occurrence of these toxins in peanut samples. A modified extraction method for CPA and aflatoxin analysis simultaneously was developed.

Materials and methods

Samples

Fifty samples of peanuts grown from the peanut-growing area in the province of Córdoba were

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analysed. Sampling was performed at the Estación Experimental Regional Agropecuaria Manfredi (Instituto Nacional de Tecnología Agropecuaria—INTA). Samples were farmers' stock, not sorted by size or quality (all grades included). Each sample (1 kg) was ground to produce a homogeneous paste and then a representative subsample of 25 g was collected for analysis.

Mycotoxin analysis

The extraction method was a modification of that proposed by Prasongsidh *et al.* (1998) for analysis of CPA in milk. The subsample of peanut (25 g) was mixed with 100 ml methanol:2% sodium hydrogen carbonate mixture (7:3). The mixture was blended at high speed for 3 min, centrifuged at 1500 rpm for 15 min and then filtered. The filtrate (50 ml) was defatted twice with 50 ml hexane by 3-min wrist action shaking, then 25 ml 10% KCl solution was added and the mixture acidified to pH 2 with 6 N HCl. The solution was transferred quantitatively to a separatory funnel, extracted twice with 25 ml chloroform and filtered over an anhydrous sodium sulphate layer. This extract was rotary evaporated to dryness and redissolved in 200 µl chloroform.

The presence of aflatoxin and CPA was determined by thin-layer chromatography (TLC) separation on silica gel G 60 plates (20 × 20 cm; Merck). TLC plates for CPA determination were immersed completely in a 2% (w/w) solution of oxalic acid in methanol for 10 min, heated at 100°C for 1 h and cooled. The plates were spotted with 2 µl of the respective extract. Standard solution was positioned every fourth track. The plates were developed in the solvent mixture ethyl acetate:2-propanol:ammonium hydroxide (40:30:20 v/v/v). After development, the plates were dried 5 min at 50°C to drive off ammonia and then sprayed with Ehrlich's reagent (1 g 4-dimethylaminobenzaldehyde in 75 ml ethanol and 25 ml concentrated HCl), with subsequent development of blue spots. Evaluation was performed after 10 min of colour development by visual comparison and densitometrically by absorbance at 546 nm with a Camag TLC Scanner III. In positive samples, CPA was confirmed by absorption spectrum of the compound without derivatization (absorption maximum 280 nm) and of the CPA 4-dimethylaminobenzaldehyde derivative at 546 nm. In addition, a confirmatory test based on two-dimensional TLC was performed (Matsudo and Sasaki 1995).

Aflatoxin plates were developed in chloroform: acetone (90:10) and evaluated under longwave UV light (366 nm) by visual comparison with standards. The presence of AFB_1 and AFG_1 was confirmed by developing the plate in another solvent system (toluene:ethyl acetate:formic acid, 5:4:1) and also by AOAC Official Method 975.37 (AOAC International 1995).

The experimental detection limit of the method was 50 µg kg^{-1} for CPA and 1 µg kg^{-1} for aflatoxins. The method was assessed on peanut samples spiked simultaneously with CPA and AFB_1 at the same level (100, 200 and 500 µg kg^{-1} for each toxin). Recovery at each level was expressed as the mean of five replicates. The relative standard deviation (RSDr) was calculated for each spiking level to give a measure of the precision of the method. The overall recovery (%) was determined by a linear regression method.

Results and discussion

The analytical method showed a good performance for CPA detection through the analysis of the spiked peanut samples at the different levels of concentration (table 1). Recoveries were lower for AFB_1, probably due to the step of alkaline extraction (methanol:2% sodium hydrogen carbonate, 7:3). However, it was observed that this extraction system was necessary to increase recovery of CPA. Acidic extraction with methanol:85% phosphoric acid was also assessed, showing lower recoveries for both toxins. Taking into account its analytical attributes, the method is considered acceptable as screening method for the simultaneous detection of both toxins in order to evaluate their natural co-occurrence.

From 50 peanut samples analysed, two were naturally contaminated with both CPA and aflatoxins. Table 2 shows the level of contamination in the naturally contaminated samples. The frequency of contamination with both toxins is relatively low but levels of contamination are similar to those reported by other authors. In an early report about CPA as a natural contaminant in peanuts, Lansden and Davidson (1983) found this toxin in 21 of 27 loose-shell kernel fractions at a range of 32–6530 µg kg^{-1} and in four of 21 sound mature kernel fractions at 32–130 µg kg^{-1}.

Table 1. Recovery and precision results for analysis of CPA and AFB_1 in peanuts.

Level spiked ($\mu g\,kg^{-1}$)	CPA[a]			AFB_1[a]		
	Found ($\mu g\,kg^{-1}$)	Recovery (%)	RSDr (%)	Found ($\mu g\,kg^{-1}$)	Recovery (%)	RSDr (%)
500	466	93.2	9.82	316	63.1	22.1
200	199	99.3	5.08	133	66.7	31.1
100	77.3	77.3	9.07	62.4	62.4	10.6

[a] Five replicates.

Table 2. Concentration of individual toxins in the contaminated samples.

Sample	CPA ($\mu g\,kg^{-1}$)	Aflatoxins ($\mu g\,kg^{-1}$)	
		B_1	G_1
1	4300	625	625
2	493	435	83

Different to the present study, their results indicated that CPA contamination may occur without any detectable aflatoxin formation. Urano *et al.* (1992) found CPA in 51% of 45 corn samples contaminated with 25–2800 $\mu g\,kg^{-1}$ (average 467 $\mu g\,kg^{-1}$) and 87% contaminated with aflatoxin (1–2300 $\mu g\,kg^{-1}$, average 252 $\mu g\,kg^{-1}$). Martins and Martins (1999) found lower ratios of these mycotoxins in mixed feeds (6.2% of 80 samples contaminated with 160 $\mu g\,kg^{-1}$ of CPA and 45% with AFB_1, from 1 to 16 $\mu g\,kg^{-1}$).

This is the first report of natural occurrence of CPA in peanuts grown in Argentina. Sample 1 was contaminated with a relatively high concentration of CPA, in conjunction with AFB_1 and AFG_1. These results should prompt a wider study of natural occurrence of this metabolite in peanuts cultivated in Argentina, where peanut meal is used as a raw material in poultry feeds. It can be hypothesized that CPA could be involved in some pathologies of unknown aetiology observed in chickens. Some of the symptoms reported by veterinarians are similar to those described when this toxin was studied on laboratory animals, e.g. erosion in the gizzard and opisthotonus, a characteristic posture adopted by the birds when they die which, according to Cole (1986), is a very good diagnostic feature of an acute dose of CPA in chicken (single oral dose of 12 $\mu g\,kg^{-1}$ body weight). As it has been demonstrated that CPA accumulates in the muscle of chicken following oral dosing (Norred *et al.* 1988), a potential for contamination of meat and meat product exists. Additional investigations to determine CPA concentration in feeds and foods, including meat, milk and eggs, should be completed in order to assess the risk of human and animal exposure to this toxin.

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