High Levels of Ochratoxins A and B in Moldy Bread Responsible for Mycotoxicosis in Farm Animals

Investigations were carried out on naturally moldy bread causally associated with a mycotoxicosis characterized by lethal gastroenteritis in poultry, rabbits, and dogs. A sample of moldy bread was found contaminated by 80 mg of ochratoxin A and 9.6 mg of ochratoxin B per kg of dry bread and colonized by *Aspergillus ochraceus*. Ochratoxins were determined by high-performance liquid chromatography. Their identification was performed by spectroanalytical data as well as by derivative preparation. Analysis of the same sample for aflatoxins, patulin, penicillic acid, and citrinin gave negative results. This is the first instance of the natural occurrence of ochratoxin B in bread; moreover, for the first time ochratoxins were revealed in foodstuffs in Italy. Considerations are made on mycotoxicological risks associated with the indiscriminate use of waste urban foods as feedstuffs.

Ochratoxins are toxic metabolites elaborated by species of *Aspergillus* and *Penicillium*. Ochratoxin A presents pronounced nephrotoxic properties on all species of animal studied and has long been recognized as a causal determinant of field cases of nephropathy in farm animals (Krogh, 1977; Scott, 1977; WHO, 1979). The toxin has been found in foodstuffs in many countries in Europe and North America, while ochratoxin B (less toxic than ochratoxin A) has been encountered only in two circumstances as a natural contaminant (Krogh, 1977). The many aspects of similarity between Balcanic endemic nephropathy (a cronic renal disease in man) and ochratoxin A induced porcine nephropathy, in addition to the high food contamination frequency in Balcanic areas (Krogh, 1977), suggest that ochratoxin A could be a nephrotoxin in man.

In this communication we report the results of mycological and mycotoxicological investigations on naturally moldy bread causally associated with a mycotoxicosis occurring in rabbit and chicken farming in the neighborhood of Bari, Italy. This toxicosis appeared as acute gastroenteritis, causing the death of several rabbits and chickens and of two dogs.

The bread was analyzed for ochratoxin A and B, aflatoxins B_1, B_2, G_1 and G_2, patulin, citrinin, and penicillic acid. Ochratoxin A, aflatoxins, and patulin have been reported as natural contaminants of moldy bread (Osborne, 1980; Hansen and Jung, 1973; Tyllinen et al., 1977), while ochratoxin B, citrinin, and penicillic acid can be produced by the same ochratoxin A producing fungi (Scott, 1977; Ciegler, 1972; Scott et al., 1972; Bacon et al., 1973).

MATERIALS AND METHODS

Loaves of moldy bread (2 × 500 g) taken from a lot of restaurant-refused bread, currently used as feed in the farm where the above-mentioned toxicosis occurred, were analyzed.

The bread, after sampling for mycological purpose, was cut in small slices, dried at 60 °C for 48 h, and finely ground for chemical analysis.

Mycological identifications were carried out by the method of Raper and Fennell (1965) using different species of the genera *Aspergillus* from CBS and NRRL collections as the reference.

Semiquantitative analysis of ochratoxins was carried out by thin-layer chromatography (TLC) after extraction with $CHCl_3$–aqueous H_3PO_4 followed by cleanup on aqueous $NaHCO_3$–diatomaceous earth and elution of toxins with $CHCl_3$–HCOOH (AOAC, 1980). A reference solution of ochratoxin B was obtained from 50 g of the same bread separately extracted according the above procedure and furtherly purified by preparative layer chromatography on silica gel with C_6H_6–CH_3OH–HCOOH (18:1:1) (eluent A). The fluorescent band appearing around R_f 0.4 under UV light at 365 or 254 nm was eluted with C_6H_6–HCOOH (99:1) and brought up in 5 mL of the same solvent. The ochratoxin B concentration in such solution was estimated by considering its molar absorption coefficient at 320 nm, ϵ = 6000 (AOAC, 1980). Spectrophotometric measurements were carried out on a Beckman spectrophotometer, Acta VI. Mass spectra were performed by a double-focusing VG-Organics ZAB 2F mass spectrometer.

High-performance liquid chromatography (HPLC) was used for the confirmation and quantitation of ochratoxins. The HPLC apparatus was a Perkin-Elmer Series 3B microcomputer-controlled pump module in connection with a P.E. LC-75 ultraviolet variable-wavelength detector set at 330 nm and with a P.E. MPF-44B fluorescence spectrophotometer equipped with a flow microcell (excitation λ 343 nm; emission λ 465 nm), in series. Fluorescence measurements were also performed on the eluting toxins from the HPLC column by stopping the flow at the related retention times. A Hibar prepacked column (250 × 4 mm i.d.) Li-Chrosorb RP-18 (7 μm) was used at 35 °C. HPLC-grade CH_3OH–H_2O (75:25) plus 1% concentrated HCOOH was employed as the mobile solvent at a flow rate of 1 mL/min.

Preparation of methyl ester derivatives of ochratoxin A and B in bread extracts was performed with 14% BF_3 in CH_3OH as elsewhere reported (Cantafora et al., 1982). The derivatized extracts were also analyzed by TLC and HPLC.

An aliquot of the $CHCl_3$–aqueous H_3PO_4 extract for ochratoxin analysis was used for extraction of citrinin and penicillic acid according the Nelson et al. (1980) procedure. TLC detection of such toxins were performed with eluent A as well as with $CHCl_3$–CH_3OH–C_6H_{14} (64:1:35). The methods for the analysis of aflatoxins B_1, B_2, G_1, and G_2 (Bottalico and Lerario, 1979) and patulin (Pohland and Allen, 1970) have been elsewhere described.

RESULTS AND DISCUSSION

The examined bread was highly contaminated by *Aspergillus ochraceus* Wilhelm, which was largely diffused in the whole loaf bread. Some small colonies of nonidentified species of the genera *Penicillium* were also present.

Aflatoxins, patulin, citrinin, and penicillic acid were not detected in the sample of moldy bread. Ochratoxins A and B were present at very high levels: 80.0 and 9.6 mg/kg of dry bread, respectively. The UV spectra of ochratoxin A and B extracted from bread presented absorption maxima at 333 and 318 nm, respectively. The difference of 15 nm between the maxima is owed to the presence of a chlorine in the ochratoxin A structure (Figure 1) (Steyn, 1971). The fluorescence emission maxima occurred at 465 (excitation maximum 335 nm) and 456 nm (excitation maximum 320

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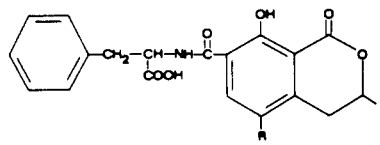

Figure 1. Chemical structure of ochratoxins A (R = Cl) and B (R = H).

nm) for ochratoxin A and B, respectively. Mass spectral data m/e in electron impact mode were 403 ($M^{+\cdot}$), 360, 255, 239, and 221 for ochratoxin A and 369 ($M^{+\cdot}$), 221, and 205 for ochratoxin B. Retention times in HPLC with the fluorometric detector (R_f values in TLC with solvent A) were 8.04 (0.46), 5.53 (0.38), 12.15 (0.75), and 7.80 min (0.51) for ochratoxin A and B and ochratoxin A and B methyl esters, respectively.

The high formation of ochratoxins in such a substrate can be easily explained by considering the storage temperature (25–30 °C) and the high moisture content of bread, which are favorable to the growing of *A. ochraceus* and, consequently, to the ochratoxins' elaboration (Ciegler, 1972; Tyllinen et al., 1977; Haeggblom, 1982).

This is the first report of the natural occurrence of ochratoxin A and B in Italy, and the first time that ochratoxin B is revealed in bread. The presence of ochratoxin A and B in such high concentrations in foodstuffs has never been reported, the highest levels encountered at present being 27.5 ppm of ochratoxin A and traces of ochratoxin B in plant products (Krogh, 1977). The concentration of ochratoxins in the examined sample is remarkably higher than lethal doses for animals affected by the considered mycotoxicosis. In fact, a diet containing less than 10 mg of ochratoxin A/kg of body weight can cause the death of poultry and dogs in a few days (Peckham, 1977; Carlton and Szczech, 1977). Moreover, the translocation of ochratoxins into the meat of animals fed on such contaminated bread is to be expected with a consequent potential risk of intoxication in man.

Although this is a single incident, the high contamination found indicates that the use of moldy bread for animal nutrition represents a very threat. Therefore, it is stressed once more the necessity of the utmost care in diverting into animal feeds waste foods, especially bread, since it has a tendency to become moldy because of its high moisture content.

Registry No. Ochratoxin A, 303-47-9; ochratoxin B, 4825-86-9.

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Angelo Visconti
Antonio Bottalico*
Istituto tossine e micotossine da parassiti vegetali
Consiglio Nazionale delle Ricerche
Via Amendola 197/F
70126 Bari, Italy

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Uptake and Persistence of Metalaxyl in Sunflower Plants

In this paper the results of a study of the translocation and persistence of Metalaxyl in sunflower plants grown under controlled conditions are reported. The active principle, which is very effective in the control of *Plasmopara helianti*, was applied both as a seed dressing and by incorporation into the soil. Quantitative analysis were performed on roots, stems, and leaves by extraction of the active principle with methanol, purification by sweep codistillation, and gas chromatography with a nitrogen–phosphorus detector. Mass spectrometry coupled with gas chromatography was used to confirm the identity of the compound. The lowest limit of sensitivity was 0.01 ppm. The results showed rapid translocation of the fungicide toward the upper parts of the plant, as indicated by the high concentration found in the leaves a few days after planting. Although the concentration showed a steady decline after reaching its maximum, Metalaxyl was still present in the leaves 90 days after planting.

New possibilities to control plant diseases have become available with the discovery of systemic fungicides that are active against ficomycetes. Metalaxyl [DL-*N*-(2,6-dimethylphenyl)-*N*-(2-methoxyacetyl)alanine methyl ester] is one of these active principles, which allows the control of one of the more serious sunflower diseases: downy

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