

## Ochratoxin A: Isolation and Subsequent Purification by High-Pressure Liquid Chromatography

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Received for publication 31 July 1978

A purification method for ochratoxin A, using liquid-liquid extractions and a final cleanup by high-pressure liquid chromatography, is described.

We have previously reported development of a solid-substrate fermentor suitable for multi-gram-quantity production of ochratoxin A (OTA) (6). However, although ochratoxin yields were 2.5 to 4 g/kg of wheat, the described methods for isolation of this toxin from small quantities of grain were designed to yield an extract for quantitative thin-layer chromatography (TLC). Other methods for extraction from liquid media were also unsuitable when applied to the larger scale we were investigating. Large quantities of OTA are needed for toxicological and other investigations on large farm animals (2, 3, 5, 7, 8).

The major portion of the fermented wheat from previous studies (6) (1973 to 1974) had been stored at  $-29^{\circ}\text{C}$  in plastic bags. Samples from all bags were reanalyzed by quantitative TLC to determine loss of OTA by storage. Most OTA activity from material originally assayed at 200 mg/kg to 4 g/kg remained within values of 250 mg/kg to 2.5 g/kg.

The extraction and purification procedure we have developed is outlined in Fig. 1. All steps were performed at room temperature under subdued light because of the sensitivity of OTA to elevated temperatures or exposure to UV light. Visual determination of TLC plates (Silica Gel 60) developed in benzene-acetic acid (90:10) was used to estimate OTA concentrations throughout the procedures (4). Final yields of crystalline OTA were determined gravimetrically.

Fermented wheat (2,274 g) placed (1-kg batches) in a 1-gallon (ca. 3.8-liter) Waring blender with an equal weight of distilled water was blended for 1 min. To this macerate 8 liters of methanol was added, and the suspension was stirred overnight with an air-driven stirrer. After filtration, the solids were suspended in 7.5 liters of methanol and stirred for 4 h. The combined filtrates (18,500 ml) were then stirred overnight with 3.8 liters of methylene chloride. To this mixture, 6 liters of 3 N sodium chloride was

added, forcing the OTA into a separate methylene chloride phase. The methylene chloride was removed (batchwise) in a 2-liter separatory funnel, and a second portion (2 liters) of methylene chloride was stirred with the methanolic extract to remove any remaining OTA. The combined methylene chloride extracts (3,760 ml) were then stirred with 4 liters of 0.2 M sodium bicarbonate for 4 h. The sodium bicarbonate layer was removed and stirred with 1.5 liters of methylene chloride while the pH was adjusted to 2.5 with concentrated hydrochloric acid. Methylene chloride was removed and filtered through 200 g of anhydrous sodium sulfate to remove the remaining water. The sodium sulfate was washed with 400 ml of methylene chloride. The combined methylene chloride extracts (1,750 ml) were evaporated to dryness under partial vacuum. The residue was shaken with 400 ml of ethyl acetate and then filtered. The filtrate was again evaporated to dryness, and the residue was dissolved in a minimal amount of ethyl acetate (total volume, 32 ml).

A model 100 Waters high-pressure liquid chromatograph with a Porasil A column (7 mm by 122 cm) was used for separations. Gradient elution was carried out with a model 660 programmer module and two model 6000 pumps. All separations were made with a 1-h linear program, beginning with 10% ethyl acetate in *n*-hexane and ending with a concentration of 20% ethyl acetate in *n*-hexane. After each run was completed, the column was washed and equilibrated with ethyl acetate (10 min), methanol (20 min), ethyl acetate (10 min), and, finally, *n*-hexane (15 min).

The 32-ml OTA concentrate was injected in 2-ml aliquots per chromatographic run. The flow rate was 4 ml/min, and 5-min (20-ml) fractions were collected. OTA was usually found (as determined by TLC) in fractions 29 to 40 before contamination from other components became noticeable. The active fractions were rinsed from

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1. Fermented wheat (2,274 g)  
↓
  2. Ground in Waring blender with equal weight of water  
↓
  3. Extracted twice with methanol  
↓
  4. Methanol solution (18,500 ml, 6.13 g of OTA)  
Sodium chloride (3 M) added  
Extracted twice with methylene chloride  
↓
  5. Methylene chloride solution (3,760 ml, 3.76 g of OTA)  
Extracted with 0.2 M sodium bicarbonate  
↓
  6. Bicarbonate solution  
Acidify to pH 2.5 with concentrated HCl  
Extract with methylene chloride  
↓
  7. Methylene chloride solution (1,750 ml, 3.5 g of OTA)  
Filtered through sodium sulfate  
Evaporate to dryness at room temperature  
↓
  8. Ethyl acetate solution  
Filtered and evaporated to dryness  
↓
  9. Ethyl acetate solution (32 ml, 3.2 g of OTA)  
Injected into high-pressure liquid chromatograph  
↓
  10. Active fractions evaporated to dryness  
↓
  11. OTA crystallized from benzene (2.7 g of OTA)
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FIG. 1. General procedure for purification of OTA from fermented wheat. The OTA yield for each step in the isolation procedure is given.

the tubes with chloroform and combined. After evaporation to dryness under a stream of N<sub>2</sub>, the residue was dissolved in 20 ml of warm benzene (35°C). OTA was allowed to crystallize from the solution at room temperature and was removed by filtration. Crystals were then washed with cold benzene. A final yield of 2.7 g of OTA was obtained, or 44% of the 6.13 g originally estimated to be present.

In another experiment, 3,000 g of wheat containing 5.18 g of OTA yielded 3.04 g of crystalline OTA. A slightly different solvent ratio was used,

and the final high-pressure liquid chromatography separation was performed with a Waters Prep LC/System 500 and the benzene-acetic acid (90:10) solvent system used for TLC. A subsequent bicarbonate extraction of the active fractions was then needed to eliminate the acetic acid from the OTA. This latter instrument permits processing of multigram quantities of toxin in a single run, unlike the semipreparative unit we used for most of these studies. However, either instrument is preferable to the less efficient and slower mode of column chromatography previously used by other investigators.

The major OTA loss in these studies occurs during the methylene chloride partition of the methanol-water extract (step 4). This extract was checked for OTA content by TLC and found to be negative. Further studies are needed to determine if a fluorescence enhancement or quenching effect during the TLC may account for the discrepancies. Limited spectroanalytical studies have been carried out on OTA (1, 9), but an expansion of these investigations would be desirable.

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