Analytical-Scale Supercritical Fluid Extraction of Aflatoxin B_1 from Field-Inoculated Corn†

Scott L. Taylor,* Jerry W. King, John L. Richard, and Judith I. Greer

Food Quality and Safety Research Unit, Mycotoxin Research Unit, National Center for Agricultural Utilization Research, Agricultural Research Service, U.S. Department of Agriculture, 1815 North University Street, Peoria, Illinois 61604

Analytical-scale supercritical fluid extraction (SFE) was applied for the removal of aflatoxin B_1 from field-inoculated corn samples. Various pressures (2000–15 000 psig), temperatures (40–80 °C), quantity of supercritical carbon dioxide (SC-CO_2) (50–500 mL of liquid CO_2), and organic modifiers were utilized to optimize the extraction method. The addition of an organic modifier to the SC-CO_2 was found to be essential to achieve high aflatoxin B_1 recoveries. Modifier additions of 5–20% by volume of an acetonitrile/methanol (2:1) mixture or neat isopropyl alcohol were tested. Analysis of extracts was performed with high-performance liquid chromatography coupled with fluorescence detection of the trifluoroacetic acid derivative. Recoveries were maximized by increasing the CO_2 pressure and organic modifier level to 10–15% (v/v). The SFE method yielded over 90% analyte recovery when compared to conventional solvent extraction procedures. Attempts to remove coextracted lipid matter by a supercritical fluid-based cleanup method are also described. The precision of the method is affected by the sample size and the homogeneity of the corn sample.

INTRODUCTION

Aflatoxins are mycotoxins produced by molds, most specifically *Aspergillus parasiticus* and *Aspergillus flavus*. The presence of these fungal toxins reduces the value of grain as an animal feed and devalues it as an export commodity (Nichols, 1983). The determination of these secondary metabolites is of much concern because of their extreme toxicity, especially their carcinogenicity. They are soluble in slightly polar solvents and insoluble in nonpolar solvents. Essentially all aflatoxins are extracted using mixtures of organic solvents such as acetone, chloroform, or methanol in combinations with small amounts of water (Bullerman, 1987). Due to the diverse nature of commodities that may be contaminated, no single method of extraction is adequate for all products (Ellis et al., 1991).

Supercritical fluid extraction (SFE) is a powerful alternative to conventional organic solvent extraction because of its combination of gaslike mass transfer and liquidlike solvating properties. To date, analytical-scale SFE has been shown to be a rapid and quantitative method for extracting relatively nonpolar components from a variety of sample matrices (King et al., 1992; Lopez-Avila et al., 1990; Wheeler and McNally, 1989). However, moderately polar compounds can be dissolved in supercritical carbon dioxide, especially at higher densities or with the addition of organic modifiers. Recently, SFE has been used as an extraction method for partially removing aflatoxin B_1 from corn (Selim and Dhawan, 1991) and peanut meal (Haas and Engelhardt, 1992).

Analysis of aflatoxin B_1 from corn and peanut samples and cottonseed products for organic solvent-based extractions requires the use of 50-g samples (AOAC, 1984a). In this study, SFE was evaluated as an alternative extraction technique for ground corn samples of 3.0–3.5 g. The goal of this research was to establish the pressure, temperature, solvent composition, and volume of CO_2 that would optimize aflatoxin B_1 extraction. The aflatoxin B_1 recoveries obtained by SFE are compared with recoveries produced by the CB method of analysis (AOAC, 1984b).

† Presented at the Midwest Section Meeting of the AOAC, June 8–11, 1992, Champaign, IL.

MATERIALS AND METHODS

Corn Samples. A single lot of corn was received and consisted of several commercial hybrids that were grown during 1990 at Union City, TN. The ears of the corn had been wound-inoculated in the late-milk to early-dough stage of kernel maturity at three equidistant points along the ear with a mixed conidial suspension of three aflatoxin-producing strains of *A. flavus* (NRRL 6412, NRRL 6444, and NRRL 3557). The corn was harvested at maturity, shelled, coarse-ground to pass a No. 5 sieve, and mixed thoroughly (sample 1). A portion of sample 1 was further ground to pass a No. 12 sieve and mixed thoroughly (sample 2).

Solvent Extraction. Corn (50 g), Celite (25 g), H_2O (10 mL), and chloroform (250 mL) were added to a 500-mL glass-stoppered Erlenmeyer flask. The flask was put on a wrist action shaker for 30 min, and then the extract was filtered through Whatman filter paper. Aliquots of the extract were taken and subjected to the CB method cleanup procedure prior to high-performance liquid chromatographic (HPLC) analysis. In addition, aliquots of the extract were analyzed without using the above cleanup procedure. Quantitation was performed using an external standard.

Supercritical Fluid Extraction. One SFE device utilized in this study was similar to a previously described unit (Favati et al., 1988) in construction and operating principle. Modifications consisted of insertion of an alumina trap prior to the compressor to purify non-helium head space welding grade carbon dioxide of trace levels of PCB-related compounds. The trap was constructed from 316 stainless steel (ss) tubing 24 in. long × 1 in. i.d. filled with 80 g of alumina C (Scientific Adsorbents, Atlanta, GA) that had been heated overnight at 300 °C. The connecting tubing between the gas booster, the extraction cell, and the micrometering (pressure reduction) valve was 0.25-in. 316 ss tubing (Autoclave Engineers, Erie, PA), pressure rated to 20 000 psi at 100 °F. Also, medium-pressure range transducers (Model 311B, Omega Engineering, Inc., Stamford, CT) with digital readouts (Model 1203, Omega Engineering) replaced Bourdon tube gauges. A commercial SFE system, the SFX 2-10 (Isco, Inc., Lincoln, NE), was also used in this study.

High-Performance Liquid Chromatography. Aflatoxin B_1 extracts were analyzed by using a modular system consisting of an SP8800 pump (Spectra-Physics Analytical, San Jose, CA), a

Rheodyne loop injector (200 μL), a Kratos Spectroflow 980 programmable fluorescence detector, and an SP4270 integrator (Spectra-Physics Analytical). The mobile phase was water/acetonitrile/isopropyl alcohol (80:10:10) pumped at 1 mL/min. A Du Pont Zorbax ODS column (4.6 mm × 250 mm, packed with 5-μm octadecylsilica) was used for the analysis. Prior to chromatography, the extracts were derivatized with trifluoroacetic acid (TFA) to convert aflatoxin B_1 to B_{2a} (AOAC, 1984c) for enhanced fluorescence detection in the reversed-phase solvents.

RESULTS AND DISCUSSION

Supercritical CO_2 is ideal for extracting relatively nonpolar, lipophilic analytes. Consequently, aflatoxins, being moderately polar compounds of intermediate molecular weight, are not ideal candidates for SFE at commonly used pressures and temperatures. It was hoped that when ultrahigh pressures (>10 000 psig) and a high extraction temperature were used, both solvation and vapor pressure effects would enhance the solubility of aflatoxin B_1 in neat SC-CO_2.

Initial extractions of sample 1 (10 g) were performed on an apparatus constructed at the National Center for Agricultural Utilization Research using neat CO_2 at high pressures. SFE conducted at 15 000 psig and 80 °C yielded the highest aflatoxin B_1 recovery. The SC-CO_2 extraction recovered 69% of the aflatoxin B_1 when compared to the recovery obtained from the CB method of analysis. However, when the SFE recovery is compared to aflatoxin B_1 recovery obtained without using the silica column cleanup of the extract, only a 56% recovery of the aflatoxin was obtained. The level of aflatoxin B_1 contamination in corn sample 1 permitted substantial dilution of the supercritical fluid extracts and circumvented the need for the cleanup step. Therefore, quantitation could be readily performed without any interference from coextracted materials.

The low analyte recoveries obtained with pure SC-CO_2 at high pressures suggested the need for an organic cosolvent during the extraction. Initially, a static SFE using 450 μL of 2:1 acetonitrile/methanol (ACN/MeOH) added directly to the extraction cell per 10 g of corn (Selim and Dhawan, 1991) followed by dynamic SFE with pure CO_2 was attempted at three different pressures. The results from extractions at 5000 and 10 000 psig at 40 °C were comparable (84 and 88 ppb, respectively) and 20 times greater than results obtained from the extraction at 1400 psig. A rationale for the low recovery of these extracts may lie in the use of an organic solvent ($CHCl_3$) in the receiver vessel, whereas the initial extractions were collected into dry round-bottom flasks. Possibly, the flow rate of the expanded carbon dioxide was impeded in the solvent-laden collection device, resulting in a lower total volume of CO_2 passed over the sample. Supercritical fluid extractions with the modifier used only 100 mL of CO_2 compared to 500 mL in the initial extractions. Therefore, lower recoveries were not unexpected.

Dynamic SFE with 2:1 ACN/MeOH modified CO_2 was attempted next with the Isco SFX 2-10 module on sample 1 (3.0–3.5 g). The extracts were collected in vials containing 10 mL of $CHCl_3$. Table I shows the results from using various pressures and temperatures while the modifier percentage and volume of CO_2 constant were held constant. These data indicate that higher pressures and higher modifier percentages will yield better recoveries of aflatoxin B_1.

Because of a positive outlier (not noted in Table I), a portion of corn sample 1 was reground and mixed (sample 2) before the next set of supercritical fluid extractions was run. Also, the reground subsample was reanalyzed by organic solvent extraction for comparison to SFE results. The new organic solvent extraction data were used for comparison purposes in Table II. The variability associated with testing corn samples for aflatoxin has been shown by Whitaker et al. (1979) and Shotwell and Stubblefield (1972). The latter study is the foundation for the official AOAC method (AOAC, 1984d) for preparing corn and soybeans for aflatoxin determination. It details that the entire sample be ground to pass a No. 14 sieve (coarse grind) and that a 1–2-kg subsample be ground to pass a No. 20 sieve (fine grind). Francis et al. (1988) reported on the aflatoxin variation in 10-g corn samples and improved upon the coarse–fine grinding procedure by comminuting the entire sample to pass a sieve opening of 0.889 mm. The precision of the aflatoxin determination is increased because the entire sample is finely ground and mixed.

Table I. SFE Screen of Aflatoxin B_1 in Corn Sample 1

pressure, psig	temp °C	modifier,[a] %	vol of CO_2, mL	recovery of B_1, ppb
2000	40	5	50	45
2000	80	5	50	5
5000	40	5	50	331
5000	80	5	50	356
7500	40	5	50	391
7500	80	5	50	304
CB method[b]				538
no cleanup[c]				660

[a] ACN/MeOH (2:1). [b] Silica column cleanup of $CHCl_3$ extract. [b] No silica column cleanup of $CHCl_3$ extract.

Table II. SFE Screen of Aflatoxin B_1 in Corn Sample 2

pressure, psig	temp °C	modifier,[a] %	vol of CO_2, mL	recovery of B_1, ppb
5000	80	5	100	476
5000	80	10	100	274
5000	80	15	100	595
5000	80	20	100	342
7500	40	5	100	446
7500	40	10	100	502
7500	40	15	100	459
7500	40	20	100	282
CB method[b]				449
no cleanup[c]				595

[a] ACN/MeOH (2:1). [b] Silica column cleanup of $CHCl_3$ extract. [c] No silica column cleanup of $CHCl_3$ extract.

To conduct the next series of extractions, the extraction parameters of 5000 psi at 80 °C and 7500 psi at 40 °C were chosen on the basis of the initial screening results (Table I). Here the modifier (ACN/MeOH 2:1) percentage and CO_2 volume were incrementally increased. The results obtained by these changes in experimental conditions are listed in Table II. These data demonstrated that the optimum modifier percentages for the above extraction conditions were 15% and 10%, respectively. Also, apparently larger volumes of CO_2 contributed to higher recoveries. The extraction performed at 5000 psi, 80 °C, 15% modifier, and 100 mL of LCO_2 (liquid CO_2) was equivalent to the organic solvent extraction without employing the silica column cleanup.

To substantiate the above data, supercritical fluid extractions at 5000 psi, 80 °C, and 15% ACN/MeOH (2:1) and using 100 mL of LCO_2 were run several times with the extracts collected in vials containing $CHCl_3$ (10 mL). Also, multiple organic solvent extractions were performed with both the 50-g sample size and additionally on 3-g samples. These extraction results are summarized in Table III. The SFE data set shows 95% recovery of the aflatoxin B_1 but has a relative standard deviation (RSD) almost twice that

Table III. SFE vs Chloroform Extraction of Aflatoxin B_1 from Corn Sample 2

method (n = 5)	av rec, ppb	norm rec, %	RSD
CB method[a]	441.4	80.4	3.2
SFE	519.6	94.6	6.2
no cleanup[b]	549.2	100.0	3.5
no cleanup[c]	515.1	93.8	10.7

[a] Silica column cleanup on $CHCl_3$ extract of 50-g sample. [b] No silica column cleanup on $CHCl_3$ extract of 50-g sample. [c] No silica column cleanup on $CHCl_3$ extract of 3-g sample.

compared to organic solvent extractions conducted on 50-g samples. However, when compared to the organic solvent extractions of 3-g samples, the SFE results gave identical results for the aflatoxin B_1 recovery and a lower RSD.

The SFE conditions mentioned above yielded excellent aflatoxin B_1 recoveries, but fats and lipids were also coextracted. Attempts were made to perform SFE with a different modifier (isopropyl alcohol) to achieve a more selective extraction of aflatoxin B_1. Isopropyl alcohol was chosen because it had been used previously for the process extraction of aflatoxin from defatted cottonseed (Watkins, 1989). The same SFE conditions (5000 psi, 80 °C) were used as before, but the modifier percentage and CO_2 volume varied. Unfortunately, these extractions did not yield high recoveries, the best aflatoxin B_1 recovery being only 55%.

Since selective extraction of aflatoxin B_1 was not achieved, attempts were made to first remove the interfering coextractives and then extract the aflatoxin. It was hoped that extraction of the lipids from the sample with neat SC-CO_2, followed by a second SFE with CO_2 modified with a 2:1 ACN/MeOH mixture to extract the aflatoxin B_1, would yield a cleaner extract for analysis. Three extractions were made using this procedure. Some aflatoxin B_1 (83 ppb) was extracted with neat CO_2, but about 3 times that amount (259 ppb) was recovered from the modified CO_2 extraction. However, the sum of the aflatoxin concentrations in these two fractions did not equal the amount initially present in the corn. Perhaps the oil in the corn sample aided the extraction of the aflatoxin, as evidenced by the lower yield of aflatoxin B_1 obtained from SFE of the lipid-free corn sample.

SFE recoveries obtained early in this study averaged 519.6 ppb, while extractions run 2.5 months later yielded an average of 426.5 ppb. Organic solvent extractions were repeated to see if the corn sample's aflatoxin B_1 content had changed during this time interval. The new organic solvent extracts yielded an aflatoxin B_1 concentration of 425 ppb, in close agreement with the SFE results. Thus, over a 2.5-month time span, the aflatoxin B_1 showed a loss of 124 ppb, or 22.6% of the original amount, even though the samples were kept in a freezer at −8 °C.

Since the two-step SFE was not successful as a cleanup technique, a sorbent trap was incorporated into the collection scheme in the hope of obtaining a lipid-free extract. In this scheme, the trap would collect the aflatoxin and lipids, allowing for sequential elution of the lipids and aflatoxin from the sorbent trap. Silica gel was chosen as the sorbent because it is utilized in the CB method cleanup procedure. As in the CB method, an initial batch of silica gel was deactivated with 1% water. A second batch was then prepared without water deactivation.

The sorbent trap was a stainless steel column containing 1.4 g of the silica. The SFE conditions were 5000 psi, 80 °C, 15% modifier (2:1 ACN/MeOH), and 100 mL of LCO_2. After completion of the extraction, the sorbent trap was removed and rinsed with solvent using an HPLC pump. Hexane (10 mL) and acetone (50 mL) were eluted through the trap, and the acetone was collected in two equal fractions. In addition to the sorbent trap, a secondary trap filled with solvent ($CHCl_3$) was employed after the sorbent trap to check for any breakthrough of the aflatoxin. The results of the sorbent trap experiments are summarized in Table IV. Breakthrough of the aflatoxin did occur, as evidenced by the large amount found in the organic solvent trap. This is probably not unexpected since the CO_2 modified at a 15% level with ACN/MeOH (2:1) would yield a very polar elutropic solvent.

Table IV. Collection of Aflatoxin B_1 from Corn Sample 2 Employing a Sorbent Trap[a]

rinse fraction	expt 1	expt 2
	Sorbent Trap	
hexane	2.5	0.4
acetone I	28.0	26.0
acetone II	0.1	0.8
	Solvent Trap	
chloroform	258.0	165.0

[a] Data in ppb. Expt 1, silica with 1% water added. Expt 2, silica with no water added.

CONCLUSIONS

In summary, optimal parameters for the supercritical fluid extraction of aflatoxin B_1 from ground corn have been established. The SFE conditions are as follows: 5000 psi, 80 °C, 15% modifier (ACN/MeOH 2:1), and 100 mL of LCO_2. However, additional method development is required to clean up the supercritical fluid extract, to permit aflatoxin B_1 analyses in the low parts per billion range. SFE, as applied to aflatoxin analysis, reduces organic solvent consumption and can also reduce the number of steps involved in the sample preparation methodology. Additional studies using SFE for the analysis of other aflatoxins and mycotoxins in different sample matrices are needed.

ACKNOWLEDGMENT

We acknowledge the assistance of Dr. D. T. Wicklow in providing the corn samples.

LITERATURE CITED

AOAC. *Official Methods of Analysis*, 14th ed.; Association of Official Analytical Chemists: Arlington, VA, 1984a; secs 26.024, 26.029, 26.055, pp 481, 485.

AOAC. *Official Methods of Analysis*, 14th ed.; Association of Official Analytical Chemists: Arlington, VA, 1984b; secs 26.026–26.030, pp 481–482.

AOAC. *Official Methods of Analysis*, 14th ed.; Association of Official Analytical Chemists: Arlington, VA, 1984c; sec 26.083, p 489.

AOAC. *Official Methods of Analysis*, 14th ed.; Association of Official Analytical Chemists: Arlington, VA, 1984d; sec 26.049, p 484.

Bullerman, L. B. Methods of Detecting Mycotoxins in Food and Beverages. In *Food and Beverage Mycology*, 2nd ed.; Beuchat, L. R., Ed.; AVI: New York, 1987; p 571.

Ellis, W. O.; Smith, J. P.; Simpson, B. K.; Oldham, J. H. Aflatoxins in Food: Occurrence, Biosynthesis, Effects on Organisms, Detection, and Methods of Control. *Crit. Rev. Food Sci. Nutr.* 1991, *30*, 403–439.

Favati, F.; King, J. W.; Friedrich, J. P.; Eskins, K. Supercritical CO_2 Extraction of Carotene and Lutein from Leaf Protein Concentrates. *J. Food Sci.* 1988, *53*, 1532–1536.

Francis, O. J., Jr.; Ware, G. M.; Carman, A. S.; Kirschenheuter, G. P. Use of Ten Gram Samples of Corn for Determination of Mycotoxins. *J. Assoc. Off. Anal. Chem.* 1988, *71*, 41–43.

Haas, P.; Engelhardt, H. Supercritical Fluid Extraction of Aflatoxin B1 from Food. *Abstracts of the 4th International Symposium on Supercritical Fluid Chromatography and Extraction*, Cincinnati, OH, May 19–22, 1992; Supercritical Conferences: Cincinnati, 1992; pp 123–124.

King, J. W.; Hopper, M. L.; Luchtefeld, R. G.; Taylor, S. L.; Orton, W. L. Optimization of Experimental Conditions for the SC-CO2 Extraction of Pesticide Residues From Grains. *J. Assoc. Off. Anal. Chem.* **1993**, in press.

Lopez-Avila, V.; Dodhiwala, N. S.; Beckert, W. F. Supercritical Fluid Extraction and Its Application to Environmental Analysis. *J. Chromatogr. Sci.* **1990**, *28*, 468–476.

Nichols, T. E., Jr. Economic Impact of Aflatoxin in Corn. In *Aflatoxin and Aspergillus flavus in Corn*; Diener, U. L., Asquith, R. L., Dickens, J. W., Eds.; Southern Cooperative Series Bulletin 279; Alabama Agricultural Experiment Station: Auburn, 1983; pp 67–71.

Selim, M. I.; Dhawan, S. K. Supercritical Fluid Extraction of Aflatoxins from Naturally Contaminated Corn. *Abstracts of the 105th AOAC International Meeting and Exposition*, Phoenix, AZ, Aug 12–14, 1991; AOAC: Arlington, VA, 1991; p 161.

Shotwell, O. L.; Stubblefield, R. D. Collaborative Study of the Determination of Aflatoxin in Corn and Soybeans. *J. Assoc. Off. Anal. Chem.* **1972**, *55*, 781–788.

Watkins, L. R. Extraction of Aflatoxin from Cottonseed Meal with Isopropyl Alcohol. Presented at the 38th Oilseed Processing Clinic, Southern Regional Research Center, ARS, USDA, New Orleans, LA, 1989.

Wheeler, J. R.; McNally, M. E. Supercritical Fluid Extraction and Chromatography of Representative Agricultural Products with Capillary and Microbore Columns. *J. Chromatogr. Sci.* **1989**, *27*, 534–539.

Whitaker, T. B.; Dickens, J. W.; Monroe, R. J. Variability Associated with Testing Corn for Aflatoxin. *J. Am. Oil Chem. Soc.* **1979**, *56*, 789–794.

Received for review December 7, 1992. Revised manuscript received April 13, 1993. Accepted April 19, 1993. The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.