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Mustafa I. Selim & Mei-Hua Tsuei

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Development and Optimization of a Supercritical Fluid Extraction Method for the Analysis of Aflatoxin B_1 in Grain Dust

*Mustafa I. Selim**

Mei-Hua Tsuei

Institute of Agricultural Medicine and Occupational Health, Department of Preventive Medicine and Environmental Health College of Medicine, University of Iowa, Iowa City, Iowa 52242.

A new method for the extraction of aflatoxins from small quantities of airborne dust, using supercritical carbon dioxide, is described. The efficacy of this method is compared with a previously used liquid extraction procedure, using naturally contaminated grain dust samples. Supercritical fluid extraction (SFE) provides faster, more sensitive, and more selective extraction, compared with the liquid extraction method. The optimized SFE conditions for 0.1–1.2 g of dust in a 2.5 mL extraction chamber are: 2000 psi, 40° C, 15 mL of liquid CO_2, static extraction for 15 min, and 250 μL of acetonitrile added to sample in the extraction chamber. A detection limit of 1 ng aflatoxin B_1 per sample is obtained using off-line HPLC analysis with UV detection. Lower detection limits are obtained using on-line SFE and HPLC analysis with fluorescence detection.

The detection of aflatoxin B_1 in grain dust represents a high health risk to farmers due to their repeated exposure during harvesting, transporting, grain storing, milling, mixing, and animal feeding operations.[1–4] Inhalation of aflatoxin-contaminated dust may be responsible for the pulmonary mycotoxicosis thought to be caused by these mycotoxins.[5–8] Recent epidemiological studies[9] and case reports[10] provide evidence for possible association between lung cancers and other types of cancer and exposure to aflatoxins through the inhalation route. However, there is no direct evidence to link the grain dust exposure and aflatoxin hazard to the rate of lung cancer, or any other cancer, among farmers.

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*Author to whom correspondence should be addressed.

Current methods for the analysis of aflatoxin in grain dust are adopted from the standard methods of the Association of Official Analytical Chemists (AOAC)[11] for the analysis of aflatoxins in whole grain. The procedure generally involves extraction with chloroform or methylene chloride followed by solid phase clean-up of the extract and analysis by thin layer chromatography (TLC) or high-pressure liquid chromatography (HPLC).[12–13] These methods are time consuming, have limited sensitivity, and require the use of large volumes of solvents, thereby creating undesirable exposure of personnel to solvent vapors and attendant disposal problems. More sensitive techniques, such as enzyme and radioimmunoassay methods, lack the selectivity needed for reliable identification of the aflatoxins. These methods are highly useful as screening techniques, but they generally require further confirmation with a different analytical system such as gas or liquid chromatography in combination with mass spectrometry. A more efficient, reliable, and highly sensitive analytical method is currently needed to measure the total yearly exposure dose from various on-farm grain handling activities.

Supercritical fluid extraction has been recently introduced as a viable alternative for liquid extraction. The term, "supercritical fluid," refers to the physical state of a substance that is maintained above its critical temperature and critical pressure. The underlying advantages of supercritical fluid extraction are based on the transport properties of the supercritical fluids; their high diffusivity, low viscosity, and adjustable liquid-like density.[14] Their high diffusivity enhances mass transfer and leads to a more efficient and rapid separation than liquid extraction. The low viscosity and absence of surface tension in supercritical fluids increases the speed of fluid penetration through the sample, so that the interaction of the solvent with the analyte molecules in the interstices of the sample matrix is enhanced. Increasing the densities of the supercritical fluids, near those of the liquid, strengthens the molecular interaction owing to shorter intermolecular

distances.[14-16] Thus, the solubility of a particular analyte in a supercritical fluid can be varied by changing the extraction pressure and temperature. This unique feature offers selective solubilization of analytes.[17-19]

The primary objective of this work was to develop and optimize an SFE method for the analysis of trace concentrations of aflatoxins from naturally contaminated grain dust samples.

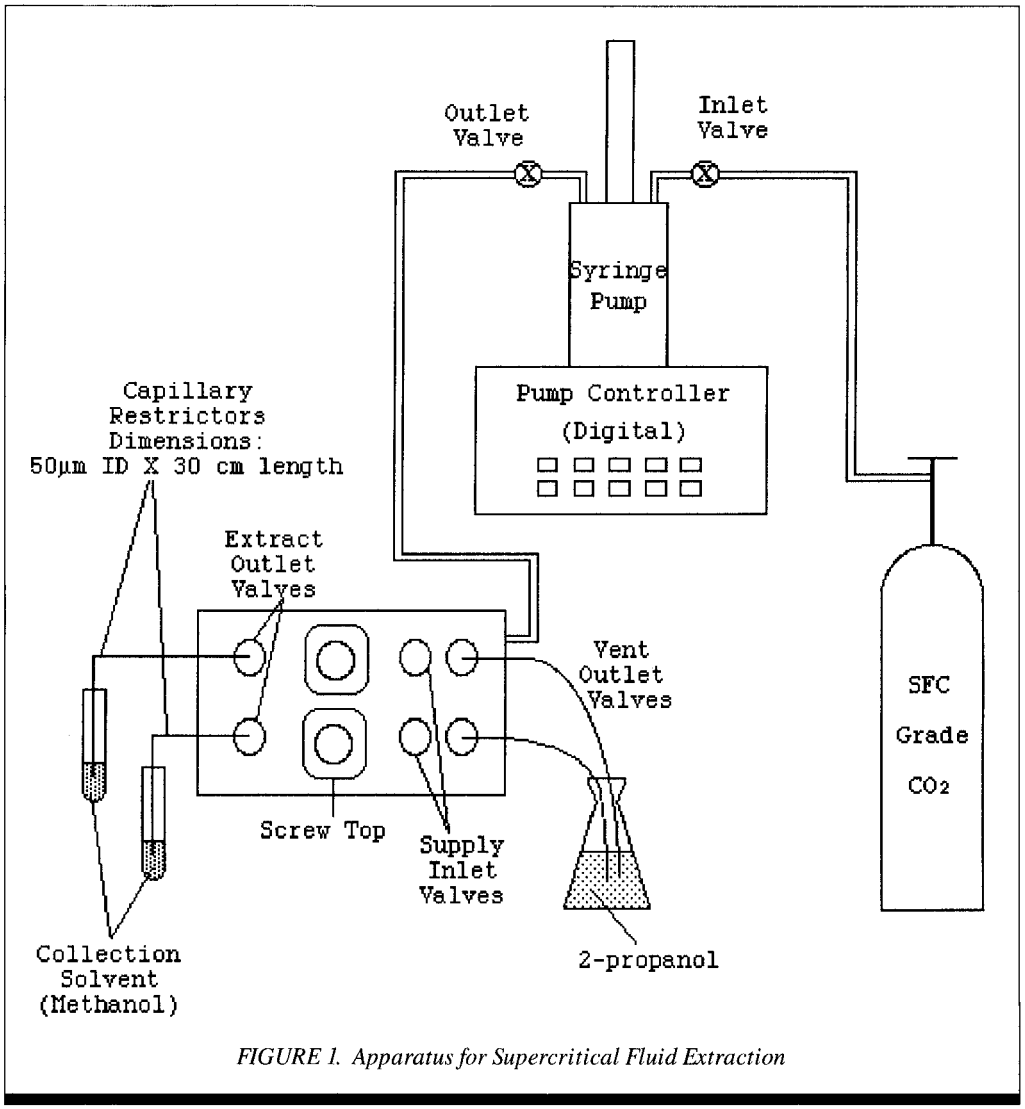

FIGURE 1. Apparatus for Supercritical Fluid Extraction

EXPERIMENTAL MATERIALS AND METHODS

Samples

Grain dust samples were collected from local farms within the state of Iowa in the winter of 1990/91. Samples were gathered from dust settled on the floors or equipment in the loading and unloading areas, storage bins, or feed-grinding areas. Most of the dust samples were from corn. All samples were stored in sealed plastic bags at −4° C. Samples were sieved through 40-mesh screen to remove large debris before the dust was analyzed.

Chemicals

1. Organic solvents were HPLC grade, purchased from Baxter Health Corporation, Scientific Products Division, (1430 Waukegan Road, McGaw Park, IL).
2. High purity liquid carbon dioxide, with 1500 psi of helium headspace and dip tube, was purchased from Scott Specialty Gases, Inc. (Plumsteadville, PA).
3. Solid aflatoxin B_1 was obtained from Sigma Chemical Company (St. Louis, MO) at 99.0% purity, and was used without further purification.
4. Solid Phase Extraction (SPE) cartridges, containing 200 mg cyano packings, were obtained from Alltech Associates (Deerfield, IL).

Classical Liquid Extraction

The liquid extraction method used is similar to those described in literature.[13,20] Approximately 2 g of dust sample was mixed with 15 g of celite and 15 mL of water, shaken with 150 mL chloroform for 30 min, and the extract was filtered through Whatman #1 filter paper. Filtered extract was transferred to a round-bottom flask, evaporated on a rotary-evaporator to near dryness, and the residue transferred to a graduated conical tube using 4x2 mL transfers in methylene chloride. Methylene chloride was then evaporated to dryness, and the residue was redissolved in 1 mL of methanol.

Extract Cleanup with Soild-Phase Extraction

The extract solution in methanol was cleaned from matrix interferences using cyano cartridges, following the procedure described in literature.[21-22] The clean extract was then analyzed by thin layer chromatography (TLC),[11,13,20,23] or high pressure liquid chromatography (HPLC),[2,10] as described below.

Supercritical Fluid Extraction

The extraction apparatus (Figure 1) consisted of two syringe pumps, ISCO model 260D and a model 100D/260D with a digital controller, and a supercritical fluid extraction (SFE) apparatus, ISCO model SFX 2-10. Each syringe pump has an inlet valve (for pump refill) and an outlet valve (for controlling fluid delivery to the extraction apparatus). The outlet flow from each pump was connected to a unidirectional valve, then to a mixing-T for combining the fluid flow from

TABLE I. Recovery of Aflatoxin B_1 from Fiber Glass Filters

Extraction Method	Spiking Level (μg/filter)	Mean Recovery (%)	Standard Deviation	Coeff. of Variance (%)
Liquid/Liquid	0.2	79.5 (n = 3)	16.7	21.0
SFE with CO_2	0.1	91.6 (n = 3)	8.5	9.2

TABLE II. Recovery of Aflatoxin B_1 from Spiked Hog Dust*

Extraction Method	Spiking Level (μg/g)	Mean Recovery (%)	Standard Deviation	Coeff. of Variance (%)
Liquid/Liquid	25	60.7 (n = 3)	6.8	11.3
SFE with CO_2	18	75.7 (n = 3)	0.6	0.8

*Hog dust sample used were tested to be free from aflatoxin.

both pumps. This combination was particularly used for on-line mixing of organic solvents (used as fluid modifiers) with liquid carbon dioxide. The SFX 2-10 system has two separate extraction chambers, each of which is equipped with three control valves: a supply valve (for controlling fluid supply); an extract collection valve connected to a capillary restrictor (for controlling the outflow of the SFE extract to the collection tube); and a vent valve (for depressurization of the extraction chamber following extraction). The capillary restrictor consisted of a piece of fused silica tubing, 0.375 mm OD × 50 μm ID × 30 cm long. The sample cartridges consisted of a stainless steel body with a choice of 0.5, 2.5, or 10 mL internal volume.

Dust samples ranging from 0.1–1.2 g were used with the 2.5 mL extraction cell. Acetonitrile is added to the sample in the extraction cell, 250 μl per sample, to improve the sensitivity and selectivity of the SFE.[24] The extraction chamber was pressurized with supercritical CO_2 and allowed to equilibrate in contact at the desired extraction temperature for a given period of time, while the extract collection valve was closed. During this equilibration step, the extracting fluid acts on the sample matrix to solubilize the analyte, and hence it is referred to as "static extraction stage." This pre-equilibration time and temperature are referred to as static extraction time and temperature, respectively.

Following the static extraction stage, the outlet collection valve on the extraction apparatus was opened and extracting supercritical fluid was allowed to flow through the capillary restrictor into a collection tube containing 1 mL methanol for trapping the analyte. The collection tube was placed into a solution of iso-propanol to prevent the collection solvent from freezing under the cooling effect of the expanding CO_2. Metal frits were routinely cleaned by sonication in methanol and dried before use to prevent cross contamination of analyte and build up of matrix particles.

HPLC Analysis

The high pressure liquid chromatography (HPLC) system used consisted of two Waters model 6000A pumps (Waters, Associates, Division of Millipore Corp., Bedford, MA) and a Hewlett-Packard diode array detector, model 1050

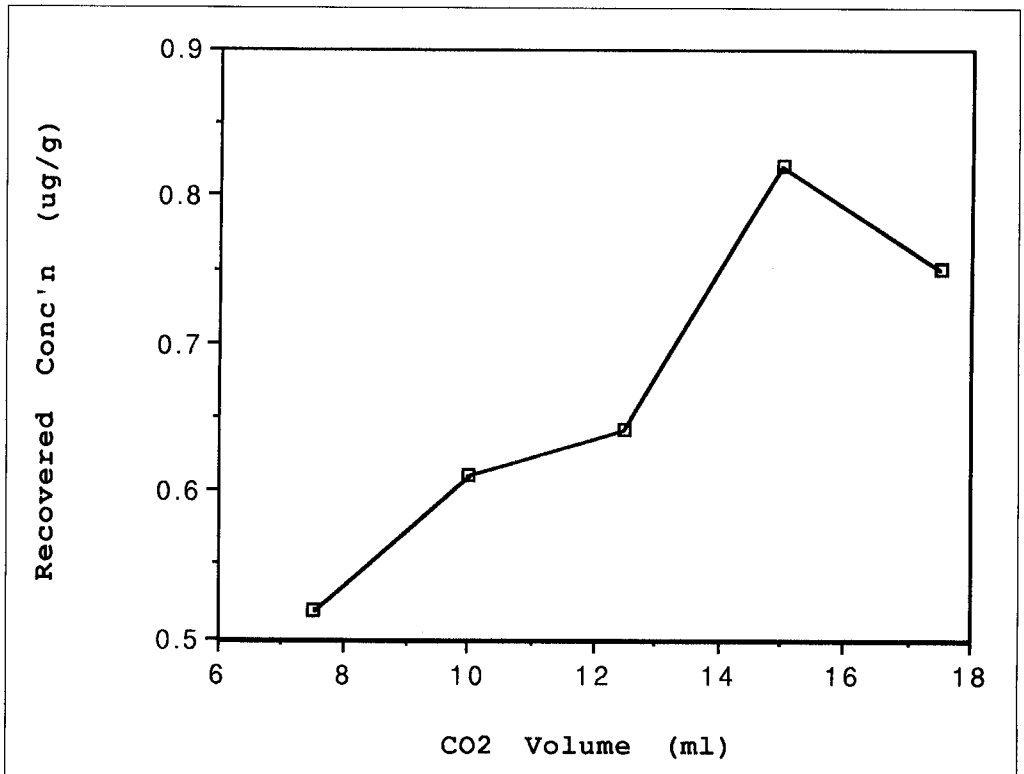

FIGURE 2. Effect of Liquid Carbon Dioxide Volume on the Supercritical Fluid Extraction of Aflatoxin B_1 From Grain Dust
Experimental Parameters:
Density = 0.658 g/mL
Temperature = 40° C
Static Extraction Time = 10 min
Spike Conc'n = 1.2 μg/g

Table III. Comparison Between SFE and Classical Liquid-Liquid Extraction of Aflatoxins From Grain Dust

Sample I.D.	Extraction Recovery (ng/g)	
	Liquid-Liquid	SFE
A121801	55.4	334.3
A121802	50.2	94.9
A121803	ND*	ND
A121804	41.6	106.7
A121801	49.5	257.0
A121802	64.6	91.0
B121803	ND	ND
B121804	ND	108.6
A010791	39.6	66.3
B010791	ND	38.3
A010891	120.0	76.8
B010891	ND	95.3
A010991	ND	ND
B010991	ND	58.6
C010991	ND	ND
AMM1114	ND	18.0
DA1114	ND	13.0
MR1114	ND	983.0

*ND: Not Detected

(Hewlett-Packard Corp., Palo Alto, CA). The analytical column was a stainless steel Supelcosil LC-18 column, 4.6 mm ID × 25 cm long (Supelco, Inc., Bellefonte, PA). Data acquisition and analysis was carried out using a Spectra Physics SP4270 computing integrator (Spectra-Physics, San Jose, CA). A mixture of water-methanol-acetonitrile (60+20 +20) was used as the mobile phase, at a flow rate of 1.1 mL/min. The UV detector was set at wavelength 365 nm, band width 8 nm, reference wavelength 450 nm and band width 50 nm, peak width > 0.85 min and response time 10 sec. At ambient temperature and the above instrumental conditions, the retention time was around 10.87 min with a retention window of ± 5%. For quantitative determination of the amount of aflatoxins in the sample, a multilevel calibration curve was constructed by plotting peak height versus the concentration of three standard solutions (2ng, 4ng, and 8ng) of aflatoxin B_1. For the HPLC system, 2ng, 4ng, and 8ng were used to calibrate the instrument detection limits.

The SFE-methanol solution was evaporated using a gentle nitrogen purge to near dryness, and the extract residue was redissolved in 0.2 mL of the HPLC mobile phase and 20 μL aliquots were injected into the HPLC. The concentration of aflatoxin B_1 was calculated from the peak height of the unknown sample and the response factor, determined from the multilevel calibration curve.

RESULTS AND DISCUSSION

Comparison of Liquid-liquid Extraction and SFE

The initial conditions used for SFE of aflatoxin B_1 from grain dust were 4000 psi pressure, 15 mL carbon dioxide volume, 10 min static extraction time, and an extraction temperature of 40° C. No modifiers were used except for the 200 μL of methanol in the aflatoxin standard solution added in the extraction cell. These conditions were used to compare the efficiency of SFE with classical liquid-liquid extraction. Table I lists the percentage recovery of aflatoxin B_1 from spiked fiber glass filters using both the liquid-liquid extraction and SFE under the above conditions.

Since the matrix composition may seriously affect the extractability of naturally contaminated matrices, an

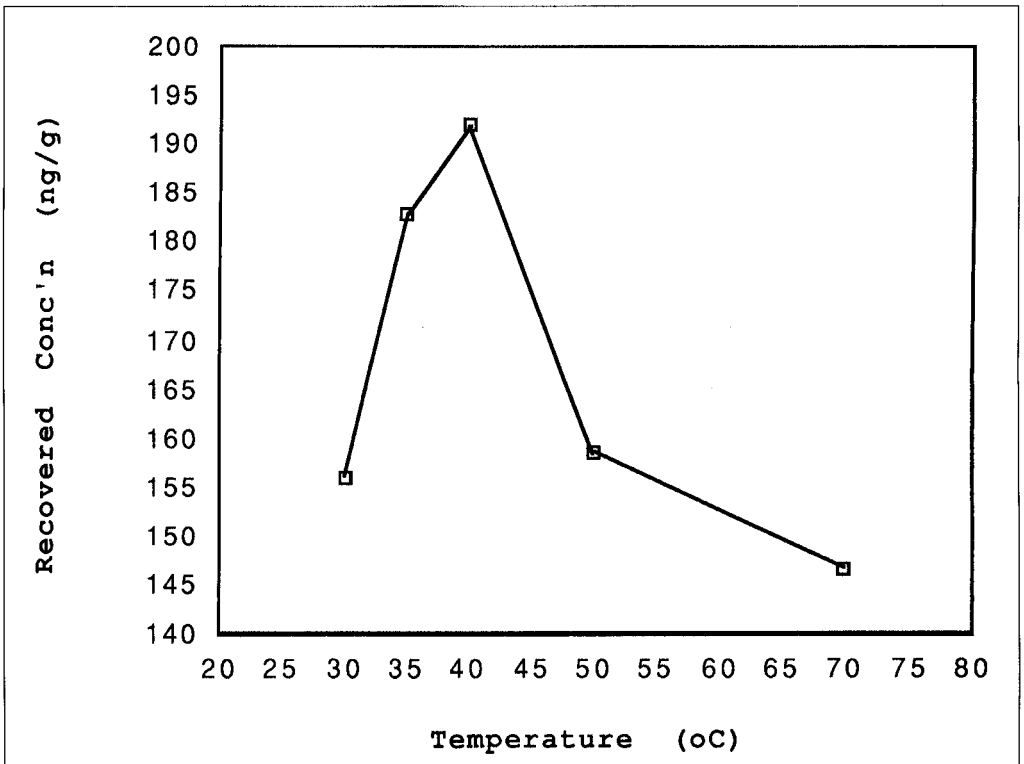

FIGURE 3. Effect of Carbon Dioxide Temperature on the Supercritical Fluid Extraction of Aflatoxin B_1 From Naturally Contaminated Dust
Experimental Parameters:
Thimble Volume = 15 mL
Pressure = 1900 psi
Static Extraction Time = 10 Min
Naturally Contaminated Dust = 0.8 g

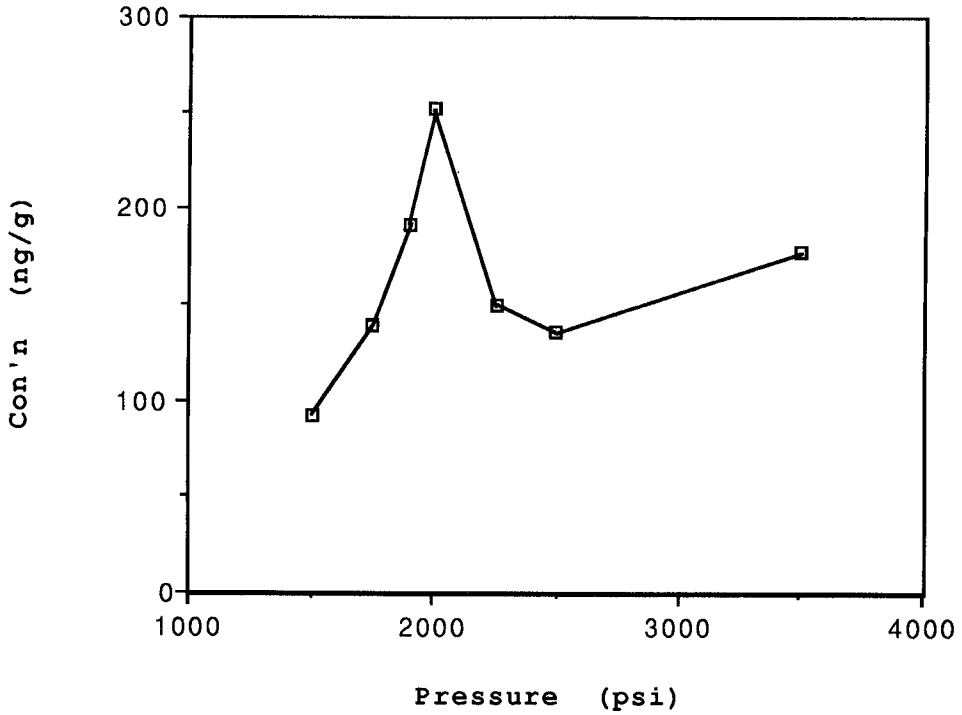

FIGURE 4. Effect of Carbon Dioxide Pressure on the Supercritical Fluid Extraction of Aflatoxin B_1 From Naturally Contaminated Dust
Experimental Parameters:
Thimble Volume = 15 mL
Temperature = 40° C
Static Extraction Time = 10 Min
Naturally Contaminated Dust = 0.8 g

aflatoxin-free hog dust sample was used as a matrix spike to compare the efficiencies of both the liquid and supercritical fluid extraction techniques. Table II lists the percentage recovery of aflatoxin B_1 from spiked hog dust samples using both classical liquid-liquid extraction and SFE. As shown in Tables I and II, the recovery and reproducibility of the SFE method are superior to the classical liquid-liquid extraction method.

The initial SFE conditions, described above, were then used to extract aflatoxins from naturally contaminated grain dust samples with one modification. 500 μL acetonitrile was added to each sample in the extraction cell. Acetonitrile was used as a modifier to improve the recovery and selectivity of the SFE method. The amounts of aflatoxin B_1 recovered using the liquid extraction and the SFE method are listed in Table III, for a set of 18 grain dust samples. With liquid-liquid extraction, aflatoxin B_1 was detected in less than half (7/18) of the samples, while with the use of SFE aflatoxin B_1 is detected in most of the samples (14/18). Although the SFE conditions used were not optimized for the recoveries shown in Tables I–III, greater aflatoxin recoveries are obtained using the SFE method. While liquid extraction requires a minimum of 2–2.5 hr per sample, SFE requires only 25–30 min and provides a cleaner extract. As a result, tremendous saving of time, solvents, and column cleanup materials are realized, compared to the liquid-liquid extraction method.

Optimization of SFE

Based on theoretical and experimental considerations of the technique,[25-26] the effect of temperature, pressure, CO_2 volume, and static extraction time were systematically investigated, in order to optimize the efficiency of the SFE for aflatoxin B_1 from grain dust. Each parameter is optimized while maintaining all others constant. For each set of experimental parameters, a minimum of three replicate extractions were made.

Carbon Dioxide Volume

In a dynamic extraction process, the sample matrix is continually exposed to a fresh stream of supercritical fluid. The movement of the analyte components from the matrix depends to a great extent on the diffusion into the mobile fluid.[15] Thus, analyte recovery is significantly affected by the total mass of carbon dioxide passed through the extraction cell. To optimize the recovery with CO_2 volume, the extraction process was carried out using 3X, 4X, 5X, 6X, and 7X volumes of carbon dioxide, where X is the volume of the extraction chamber (X = 2.5 mL, usually referred to as a thimble volume). The pressure was initially set at 1500 psi, the temperature was set to 40° C, and the static extraction time was 10 min. The mean aflatoxin recovery is plotted versus the volume of liquid carbon dioxide used as shown in Figure 2. From this figure, the highest recovery of aflatoxin is obtained using 15 mL of liquid carbon dioxide, which is six times the volume of the extraction chamber (6 thimble volumes). Lower recovery is obtained when larger volumes of liquid CO_2 are used, due to loss of aflatoxin by the bubbling action of expanding CO_2 through the collection solvent.

Effect of Temperature

The effect of temperature on the efficiency of the SFE of aflatoxin from naturally contaminated grain dust was studied at 30, 35, 40, 50, and 70° C. Three replicate extractions were carried out at each temperature, using approximately 0.8 g samples. The relative standard deviation for all measurements ranged from 2–10%, indicating good reproducibility of the experimental procedures. Figure 3 shows the plot of the average aflatoxin recovery versus the extraction temperature. The amount of aflatoxin recovered is found to increase with temperature, reaching a maximum at 40° C. At higher

temperatures, the recovery is found to decrease with increasing temperature. This behavior may be attributed to the effect of temperature on the solubility parameters for both aflatoxin and the extracting fluid, which are both functions of temperature and pressure. Increasing the extraction temperature enhances the solute solubility in the supercritical fluid due to a decrease in the solute's cohesive energy. Increasing temperature under constant pressure reduces the difference between the solubility parameters of both the solute and the extracting superitical fluid, consequently their cohesive energy is decreased leading to an enhanced solute solubility. Maximum solubility is attained when the solubility parameter for the solute is equal to the solubility parameter for the extracting supercritical fluid.[27] At temperatures greater than 40° C, the decrease in the aflatoxin solubility parameter may not be sufficient to offset the increase in the solubility parameter of the extracting supercritical fluid, resulting in a decrease in aflatoxin solubility. In addition, at higher extraction temperatures the efficiency of aflatoxin trapping in the collection solvent may be reduced leading to possible loss under the mechanical effect of the rapidly expanding CO_2 gas.

Effect of Pressure

The effect of supercritical pressure on aflatoxin recovery was studied at the optimum extraction temperature of 40° C, determined above. The recovery at each pressure was the average of 3–4 replicates with a relative standard deviation of 3–15%. Figure 4 shows the plot of the average recovery versus the pressure at 40° C. Under the conditions used, highest recovery of aflatoxin was obtained at supercritical pressure of 2000 psi. Similar maxima have been observed for other compounds in dense carbon dioxide in the pressure range of 270–1900 atm,[28] and in supercritical fluid systems, and their locations can be theoretically determined by application of regular solution theory.[27] However, these theoretical predictions are dependent on the applicability of the regular solution theory to multi-component supercritical systems.

Static Extraction Time

In the static extraction stage, the sample matrix was allowed to equilibrate with the supercritical fluid for a given period of time, under supercritical pressure and temperature. Figure 5 shows the effect of static extraction time on the amount of aflatoxin recovered from naturally contaminated dust samples. As shown in the figure, aflatoxin recovery progressively increased with increasing static extraction time, and reached maximum after approximately 15 min. Although longer extraction time is expected to either increase the extraction efficiency or maintain it at its maximum, a 30-min static extraction time resulted in lower recovery and poorer reproducibility, which in this case may be caused by chemical transformation of analyte or a system leak.

Method Detection Limit

The method detection limit (MDL) was determined following the procedure described in literature.[29] Naturally contaminated dust sample was mixed with aflatoxin free sample to form four different concentration levels of aflatoxin B_1. The optimized SFE conditions were used to obtain four replicate analyses for aflatoxin B_1 in each of the four different concentration levels. The standard deviation of the replicates was plotted versus the mean recovered concentration at the four different levels. According to American Chemical Society's Definition, MDL is arbitrarily chosen as 3 S_o, where S_o is the value of the standard deviation when the concentration of

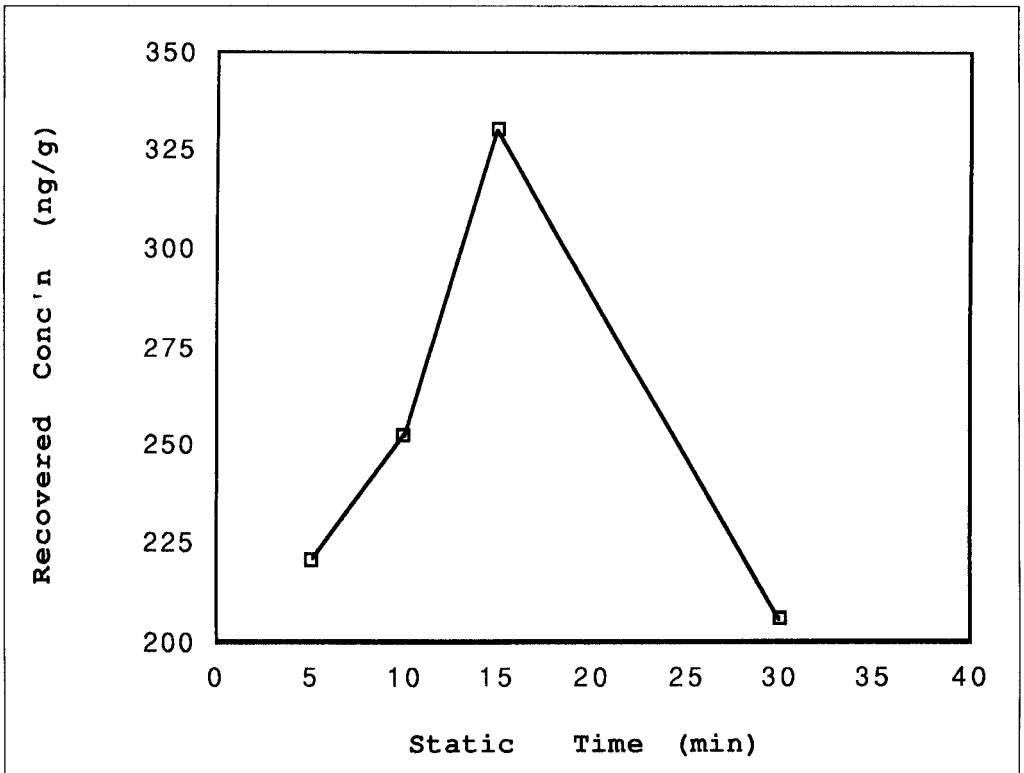

FIGURE 5. Effect of Static Extraction Time on the Supercritical Fluid Extraction of Aflatoxin B_1 From Naturally Contaminated Dust.
Experimental Parameters:
Thimble Volume = 15 mL
Pressure = 2000 psi
Temperature = 40° C
Naturally Contaminated Dust = 0.8 g

aflatoxin B_1 is zero. The calculated MDL was approximately 1 ng/g for dust samples between 0.1–1.2 g.

CONCLUSIONS

SFE provides a viable alternative for liquid extraction for many similar occupational or environmental exposure measurements. SFE appears to be faster, more sensitive, and more efficient than the classical liquid extraction procedure. It eliminates the use of and exposure to hazardous organic solvents. SFE extracts can be analyzed on-line or off-line by HPLC or HPLC/MS for improved qualitative and quantitative analysis of aflatoxin B_1 in small amounts (< 0.1 g) of dust samples.

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