Supercritical Fluid Extraction of Fumonisin B_1 from Grain Dust

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Fumonisin B_1 (FB_1) was extracted from corn, corn dust, and *Fusarium moniliforme* culture samples using supercritical carbon dioxide (SC-CO_2). The supercritical fluid extraction (SFE) conditions were optimized regarding the use of different SC-CO_2 modifiers, modifier volume, pressure, temperature, and static extraction time. The addition of the modifier both in the extraction cell (prior to the static extraction step) and on-line with liquid carbon dioxide (during the dynamic SFE step) was found to significantly increase the recovery of FB_1. Under the optimized SFE conditions (15 mL of liquid CO_2, 750 μL of 5% acetic acid per gram of sample, 1200 psi, 20 min static extraction time), the recovery of FB_1 was found to be approximately 40 times greater than the recovery using conventional solvent extraction. SFE was faster and more reproducible (RSD = 3−5%) compared to the solvent extraction (RSD = 6.5%). The calculated detection limit was approximately 150 ppb, on the basis of the HPLC analysis of FB_1−fluorescamine derivatives with UV detection.

Keywords: *Fumonisin; grain dust; supercritical fluid extraction*

INTRODUCTION

Fusarium moniliforme fungi are commonly known to contaminate corn crops worldwide (Badiali et al., 1968; Ramakrishna et al., 1989; Marasas et al., 1979; 1981; Li et al., 1980; Yang, 1980). Ingestion of *F. moniliforme*-contaminated corn has been associated with leukoencephalomalacia (LEM) outbreaks, an acute neurotoxic disease of equine (Wilson and Maronpot, 1971; Kriek et al., 1981; Haliburton et al., 1986), and porcine pulmonary edema (PPE) syndrome in swine (Kriek et al., 1981; Haliburton et al., 1986; Ross et al., 1990). In addition, *F. moniliforme* has been linked to esophageal cancer in humans in areas of the world with known high fungal contamination of food (Marasas et al., 1979, 1981; Li et al., 1980; Yang, 1980; Marasas, 1982). Isolates of *F. moniliforme*, from *Fusarium*- contaminated corn, were found to be hepatocarcinogenic (Marasas et al., 1984; Jaskiewiecz et al., 1987), nephrotoxic (Voss, 1989), and cancer-promoting in rats (Gelderblom et al., 1988).

Fumonisin B_1 (FB_1) is a secondary metabolite (mycotoxin) that has been recently isolated and characterized from *F. moniliforme* MRC826 (Gelderblom et al., 1988; Bezuidenhout et al., 1988). FB_1 was later detected in contaminated feed samples (Ross et al., 1991) associated with LEM (Wilson and Maropot, 1971; Kriek et al., 1981; Haliburton et al., 1986) and PPE (Kriek et al., 1981; Haliburton et al., 1986, Marasas, 1982; Harrison et al., 1990).

Although the health effects of FB_1 in humans are not yet established, available animal studies provide evidence for a potential health risk to farmers and grain workers from repeated low-level exposure to grain dust. However, current analytical methods for fumonisins (Gelderblom et al., 1988; Ross et al., 1991; Shepard et al., 1990) are laborious as they involve solvent extraction, extensive cleanup procedures, and concentration of the extract. Moreover, these methods are applicable to large sample size (5−25 g) and are not sufficiently sensitive for the analysis of low levels of FB_1 in corn or small samples of contaminated airborne dust.

This paper describes the development and optimization of a supercritical fluid extraction (SFE) method for the detection and quantification of FB_1 in contaminated grain dust samples.

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EXPERIMENTAL MATERIALS AND METHODS

Samples. Corn dust samples were collected from local farms within the state of Iowa in the winter of 1990−1991. Dust samples were gathered from dust settled on the floors or equipment in the loading and unloading areas, storage bins, or feed grinding areas. All samples were stored in sealed plastic bags at -4 °C. Dust samples were sieved through a 40 mesh screen to remove large debris during collection. These samples were screened to select materials in which fumonisin B_1 is undetectable. A synthetic matrix resembling naturally contaminated corn dust was then prepared by mixing "fumonisin-free" corn dust and ground corn (mesh < 1 mm) with a dried culture extract of *F. moniliforme* at 5:1 ratio, respectively.

Reagents and Materials. All organic solvents used for extraction and chromatography were of HPLC grade (Baxter Health Co., McGaw Park, IL). Fluorescamine (28 mg in 100 mL of dry acetone) and standard FB_1 (99.0% purity) were obtained from Sigma Chemical Co. (St. Louis, MO). TLC and HPLC analysis of the FB_1 standard showed that it contains fumonisin B_2 at approximately 20%. However, for the purpose of SFE optimization the FB_1 standard was used without further purification. General reagents such as acetic acid (ACS certified) were purchased from Fisher Scientific Co. (Fair Lawn, NJ). High-purity liquid carbon dioxide, with 1500 psi of helium headspace and dip tube, was purchased from Scott Specialty Gases, Inc. (Plumsteadville, PA). Water was deionized and filtered through granular activated carbon (GAC) using a Millipore Milli-Q water system (Millipore, Milford, MA). SFE extract collection and derivatization were carried out in disposable culture tubes (Corning Glass Works, Corning, NY). All other tubes or glass vials were cleaned by boiling in 35% nitric acid for 30 min followed by rinsing several times with deionized and GAC-filtered water. Metal frits were routinely cleaned by sonication in CH_3OH and dried before use to avoid any cross-contamination and buildup of matrix particles.

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Organic Solvent Extraction. A total of 1 g each of contaminated corn or dust samples was successively extracted with 2 mL (two times) of ethyl acetate and 2 mL (three times) of methanol−water (3:1 v/v), shaking each time for 30 min (Gelderblom et al., 1988). This procedure was used for the extraction of FB_1 from culture materials, and it is suited for other matrices due to the high polarity of FB_1. The methanol−water fractions were combined and evaporated to dryness. The residue was redissolved in 1 mL of methanol−water (3:1), filtered through a 0.2 mm syringe filter, and evaporated under nitrogen to 0.5 mL. The extracts were then analyzed by TLC and HPLC as described below.

Supercritical Fluid Extraction. The extraction apparatus (Selim and Tsuei, 1993) consists of two syringe pumps, an ISCO Model 260D and a Model 100D/260D with a digital controller, and a supercritical fluid extraction apparatus, ISCO Model SFX 2-10. Each syringe pump had 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 and then to a mixing-T to combine the fluid flow from both pumps. This combination was used for on-line mixing of organic solvents (used as fluid modifiers) with liquid carbon dioxide. The SFX 2-10 system had two separate extraction chambers, each 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, 52 mm i.d. X 0.375 mm o.d. X 30 cm long (from Polymicro Technologies, Phoenix, AZ). The sample cartridge consisted of a stainless steel body with a 2.5 mL internal capacity. This cartridge had a capacity of up to 0.5 g of dust. The modifier (e.g. 5% aqueous acetic acid solution) was added to the sample in the extraction cell, pressurized with SC-CO_2, and allowed to equilibrate for a period of time (static extraction time) at the desired supercritical temperature and pressure. When the static extraction time was passed, the extract collection valve was opened, allowing the extracting SC-CO_2 to pass through the capillary restrictor (maintained at 60 °C) to the collection tube containing 2 mL methanol−isooctane (1:1 v/v) to trap the analyte. The collection tube was placed into 2-propanol to prevent freezing under the cooling effect of the expanding SC-CO_2. When the SFE process was complete, the isooctane layer was removed from the collection tube and discarded. The methanol layer was evaporated to dryness, reconstituted into 250 μL of 0.1 M sodium borate buffer (pH 8.5), and derivatized with fluorescamine solution for HPLC analysis.

High-Pressure Liquid Chromatography (HPLC). The sample extract (FB_1-contaminated) was derivatized using fluorescamine (a nonfluorescent compound) to give a fluorescent derivative (Bohlen et al., 1973; Shepard et al., 1990; Samejima, 1974; Sydenham et al., 1990; Wilson et al., 1990). A 250 μL aliquot of the extract was evaporated to dryness under nitrogen, the residue was dissolved in 250 μL of 0.1 M sodium borate buffer (pH 8.5) [alternatively, 0.05 M sodium bicarbonate buffer (pH 8.6) was used (Sydenham, 1990) and 100 μL of fluorescamine was added and vigorously shaken for 30 min (Samejima, 1974; Sydenham et al., 1990; Wilson et al., 1990)]. At pH 8−9.5 the absorption maximum for the fluorescamine derivative is at 390 nm, and the fluorescence emission is at 475 nm (Bohlen, 1973).

The HPLC system used during the optimization of the SFE consisted of two Waters pumps model 6000A (Waters Associates, Millipore Corp., Milford, MA) and a Hewlett-Packard multiple wavelength detector, Model 1050 (Hewlett-Packard Corp., Palo Alto, CA). A Supelco LC-18 column, 4.6 i.d. × 15 cm, 5 mm particle size (Supelco Inc., Bellefonte, PA) was used. Data acquisition and analysis were carried out using a Spectra Physics SP4270 computing integrator (Spectra Physics, San Jose, CA). CH_3OH−0.05 M KCl (1:9 v/v) was used as the mobile phase at a flow rate of 1 mL/min, and the UV detector was set at the absorption wavelength of 390 nm.

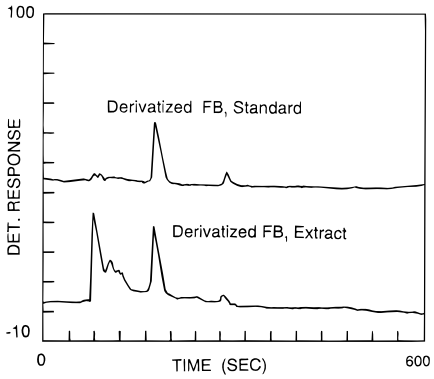

Figure 1. Sample HPLC chromatograms of FB_1−fluorescamine derivatives, sample extract and standard.

A Hewlett-Packard HPLC system Model 1090 Series II, equipped with a diode array detector, 100 × 1.5 mm, 5 μm particle size, ODS hypersil reversed phase column, was later acquired by our laboratory and used in some of the analyses for the method detection limits, as well as on-line SFE-HPLC experiments. This system was also used with a Waters model 470 scanning fluorescence detector (Waters, Division of Millipore), that was graciously loaned to us from Millipore and used for determination of the method detection limits with fluorescence detection. The excitation and emission wavelengths were set at 390 and 474 nm, respectively.

Thin Layer Chromatography (TLC). Both normal and reversed phase TLC were used for screening and detection of FB_1 in all of the samples extracted under the SFE conditions. The extracts were reconstituted in 0.5 mL of CH_3OH−H_2O (3:1 v/v), spotted on precoated analytical silica gel 60 TLC plates (0.25 mm × 20 × 20 cm), developed after saturation in a solvent mixture consisting of $CHCl_3$−CH_3OH−CH_3COOH (6:3:1 v/v). The spots were made visible by spraying with either fluorescamine solution (1% in dry acetone) (Felix and Jimenez, 1974) or 0.5% p-anisaldehyde in CH_3OH−H_2SO_4−CH_3COOH (85:5:10) and heating at 110 °C for 5 min (Sydenham et al., 1990; Wilson et al., 1990). FB_1 appeared as yellow fluorescent or as brown−purple spots with each reagent, respectively, and with an R_f value of 0.25. For reversed phase TLC screening, the sample extract was dried and redissolved in CH_3CN−H_2O (1:1). The TLC plates, Whatman KC18, were spotted with an aliquot of the extract, developed in CH_3OH−1% aqueous solution of KCl (3:2 v/v), dried, and sprayed with 0.1 M sodium borate buffer (pH 8.5) followed by fluorescamine solution (100 mg/mL CH_3CN). After 1 min, the plate was dried and sprayed with CH_3CN−0.01 M boric acid (4:6 v/v) and air-dried. FB_1 and FB_2 were visualized as bright greenish yellow spots under long-wavelength UV light. The R_f values for FB_1 and FB_2 were 0.5 and 0.1, respectively, consistent with reported literature values (Rottinghaus et al., 1992).

RESULTS AND DISCUSSION

TLC and HPLC analysis of the fumonisin standard showed a minor component of fumonisin B_2 (FB_2). However, for the purpose of SFE optimization, only FB_1 was assayed. Figure 1 shows the HPLC chromatogram for the derivatized fumonisin (FB_1 and FB_2) standard and a derivatized sample extract. FB_1−fluorescamine (FB_1-fluorophore) gave one peak with a retention time around 2.90 min, with a retention window of ±5%. A second small peak for FB_2 was also detected around 4.90 min. Although the fluorescamine derivatives are strongly fluorescent and commonly used in conjunction with fluorescence detection, in the absence of such a detector, the UV detector set at the absorption maximum of 390 nm was found to provide good sensitivity. At the alkaline pH used (pH 8.5), the FB_1−fluorescamine derivatives were found to be stable for several hours at

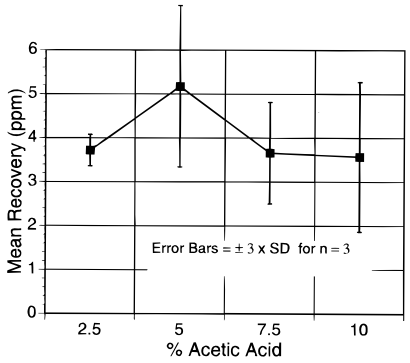

Figure 2. Effect of acetic acid concentration on the extraction of FB_1.

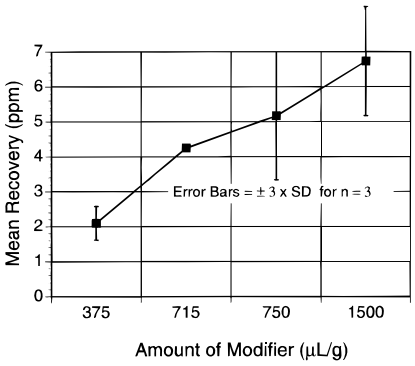

Figure 3. Effect of the amount of modifier on the recovered concentration of FB_1.

room temperature and for a few days when stored in amber vials and refrigerated (Bernardo et al., 1974).

The initial SFE conditions used for the extraction of FB_1 from corn and corn dust samples were as follows: 3000 psi, 40 °C, 15 mL of liquid CO_2, and 10 min of static extraction time. Due to the high polarity of FB_1, the effect of adding small quantities (0.5 mL) of polar solvents (SC-CO_2, e.g. methanol, acetonitrile, water, and 1% acetic acid in water) to SC-CO_2 was studied individually. The highest recovery of FB_1 was obtained using 1% acetic acid in water. The effect of acetic acid concentration was then studied by adding 0.5 mL of different concentrations of acetic acid (2.5, 5, 7.5, and 10% acetic acid in water) to the extraction cell under the above SFE conditions. Figure 2 is a plot of the FB_1 recovery versus the concentration of acetic acid. The highest recovery of FB_1 was obtained using 5% acetic acid.

The effect of the modifier volume on the extraction efficiency was studied under the above SFE conditions (3000 psi, 40 °C, 15 mL of liquid CO_2, and 10 min of static extraction time). Figure 3 is a plot of the FB_1 recovery versus the volume of modifier added to the sample in the extraction cell (milliliters per gram of sample). The recovery of FB_1 was found to increase progressively with increasing volume of modifier added. However, as shown in Figure 4, the extraction of interfering components also increased, which may limit accurate quantification of FB_1. The addition of 750 μL of 5% acetic acid to the sample in the SFE cell was found to produce the highest recovery without compromising extract cleanliness, due to coextracted interfering components.

The effect of the SC-CO_2 pressure on the recovery of FB_1 was studied under the above SFE optimized conditions and using 750 μL of 5% acetic acid/g as a modifier. Figure 5 shows the plot of the average recovery versus

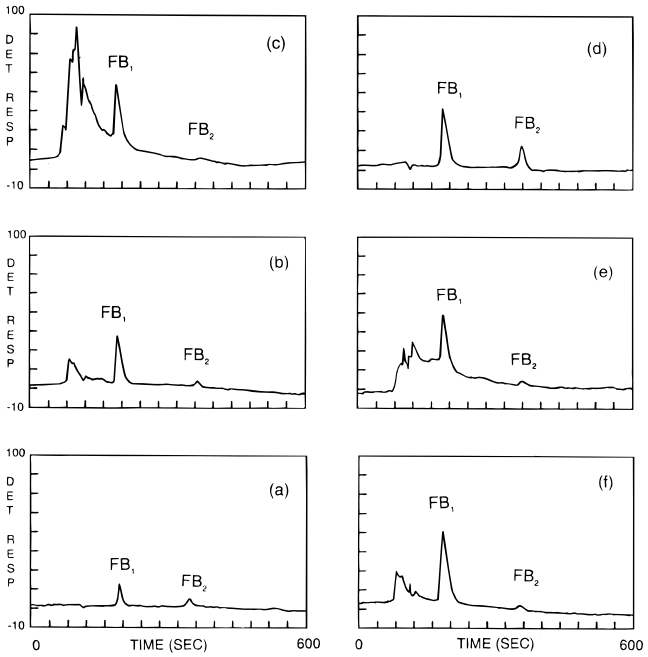

Figure 4. Sample chromatograms showing the effect of coextracted interfering components during modifier volume (b and c) and pressure (c−e) optimization.

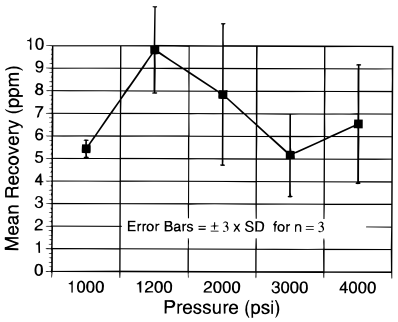

Figure 5. Effect of pressure on the SFE of FB_1.

the pressure under the above extraction conditions. The recovery at each pressure is the arithmetic mean of three or four replicate measurements with relative standard deviations (RSD) between 3 and 13%. Maximum recovery of FB_1 was obtained at a supercritical fluid pressure of 1200 psi. Similar maxima have been observed for other compounds in dense carbon dioxide and in other supercritical fluid systems (Selim and Tsuei, 1993; Giddings et al., 1970). These maxima can be theoretically determined by application of regular solution theory to a multicomponent supercritical system (King, 1989).

The effect of the supercritical fluid temperature on the FB_1 extraction was studied under the newly optimized SFE conditions (1200 psi, 750 μL of 5% acetic acid, 15 mL of liquid CO_2, and 10 min of static extraction time) (Table 1) A plot of the mean recovery of FB_1 at 40, 60, 80, and 100 °C is shown in Figure 6. The plotted concentrations are the arithmetic mean of three replicate SFE measurements at the corresponding temperature. The % RSD values for these measurements were 11.8, 3.3, 1.5, and 4.2 at 40, 60, 80, and 100 °C, respectively. The recovered FB_1 concentration was found to increase with temperature up to 60 °C (5.8 ppm) and then decrease to 4.9 and 4.1 as the temperature increased to 80 and 100°C, respectively. This could be attributed to different effects of temperature

Table 1. Summary of SFE Optimization Parameters

parameter	results	optimum parameter
CH_3COOH concn	Figure 2	5% CH_3COOH in H_2O
modifier vol (μL/g of sample)	Figure 3	750 μL/g of sample
sample size in 2.5 mL cell (g)		0.25 g
temp (°C)	Figure 6	60 °C
pressure (psi)	Figure 5	1200 psi
static extraction time (min)	Figure7	20 min
method of modifier addition	Table 2 Figure 4b,c Figure 8	in cell + on line

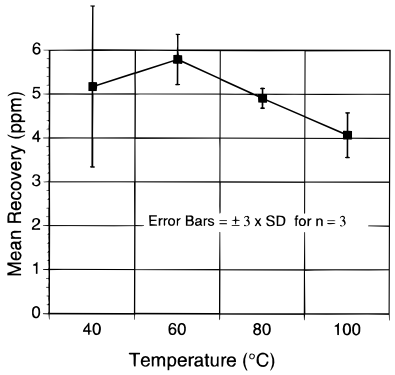

Figure 6. Effect of temperature on the SFE of FB_1.

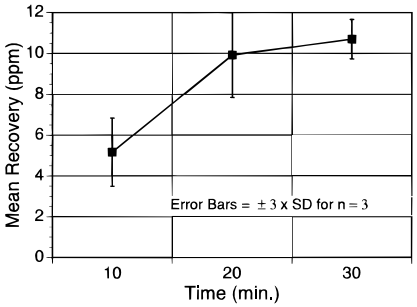

Figure 7. Effect of static extraction time on the SFE of FB_1.

on the solubility parameters of both FB_1 and the extracting fluid. Increasing the extraction temperature to 60 °C, under constant pressure, enhances the FB_1 solubility in the supercritical fluid. This is due to the decrease in FB_1 cohesive energy and the reduction of the difference between the solubility parameters of both FB_1 and the extracting supercritical fluid; consequently, their cohesive energy is decreased, leading to solubility improvement. The maximum solubility should be obtained when the solubility parameter of the FB_1 is equal to the corresponding parameter of the extracting supercritical fluid (King, 1989). At temperature over 60 °C, the decrease in FB_1 solubility parameter may not be sufficient to offset the increase in the solubility parameters of the extracting supercritical fluid, resulting in a decrease in FB_1 solubility.

Increasing the static extraction time was found to increase the recovery of FB_1 under the previously optimized SFE conditions (1200 psi, 60 °C, 750 μL of 5% acetic acid/g of sample, and 15 mL of liquid CO_2). As shown in Figure 7, FB_1 recovery increased from 5.2 ppm at 10 min to 9.9 and 10.7 ppm at 20 and 30 min of static extraction time, respectively. However, the slight increase in recovery at the higher static extraction time was again associated with an increase in the extraction of more interfering compounds that may affect reliable quantification. Therefore, 20 min of static extraction time was considered the optimum static extraction time under the above SFE conditions. Further FB_1 extraction is limited by its solubility in supercritical CO_2 and the mass transfer process.

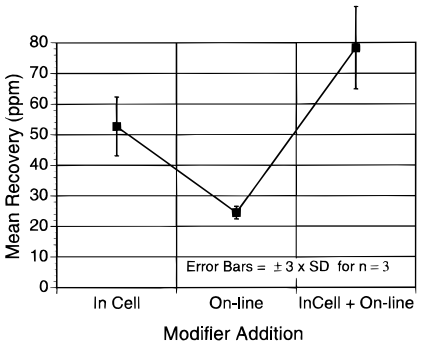

Figure 8. Effect of modifier addition (on-line and in cell) on the SFE of FB_1.

Table 2. Effect of Adding Modifier (750 mL of 5% of Acetic Acid/g of Sample) on the SFE of FB_1 from Naturally Contaminated Dust Samples

modifier addition	replicate wt (g)	measured concn (ppm)	mean concn (ppm)	% RSD
added in cell	0.2533	50.45		
	0.2539	51.16	52.7	6.1
	0.2534	56.38		
mixed on-line with liquid CO_2	0.2672	25.30		
	0.2528	23.97	24.5	2.8
	0.2250	24.34		
half added in cell plus	0.2692	83.32		
half mixed on-line with liquid CO_2	0.2622	78.22	78.3	5.7
	0.2519	78.92		
	0.2401	72.55		

Table 3. Liquid−Liquid Extraction vs SFE of Fumonisin from Naturally Contaminated Sample

extraction method	no. of replicates	mean recovered concn (ppm)	% RSD
liquid−liquid extraction	4	2.12	6.46
SFE before optimization	3	24.53	2.79
SFE after optimization	4	78.16	5.66

The addition of modifier was found to be most effective when added in cell and on-line with liquid CO_2. The effect of modifier addition rate and method FB_1 extraction from a naturally contaminated sample is provided in Table 2. The addition of the acetic acid to the sample matrix in the cell allows greater solubilization of FB_1 during the static SFE step. During the static extraction step, the modifier added to the cell penetrates the sample matrix, allowing greater solubilization of FB_1 under the SC-CO_2 pressure. Solubilized FB_1 is removed from the cell during the first few minutes of the dynamic extraction process. Further extraction of FB_1 is achieved by interaction with the acetic acid added on-line during the dynamic SFE step. As shown in Figure 8, a substantial increase in FB_1 recovery was obtained by the combined addition of the modifier (acetic acid) both in the cell (prior to the static extraction step) on-line (during the dynamic extraction step). FB_1 recoveries obtained with the liquid−liquid extraction method and SFE, before and after optimization, are provided in Table 3. The better reproducibility and much greater recoveries are obtained with the SFE method.

The theoretical method detection limit (MDL) is defined as 3 times the standard deviation at zero concentration (S_0) (American Chemical Society, 1980, 1983; Taylor, 1988). To determine the method detection limit, three concentration levels of synthetic samples of FB_1 were prepared by mixing different proportions of

dried *F. moniliforme* with a diluent containing no detectable level of fumonisin. The diluent consisted of ground corn and corn dust of mesh size <1 mm. Each concentration level was extracted in triplicate using the optimized SFE conditions (15 mL of liquid CO_2, 750 μL of 5% acetic acid/g of sample, 1200 psi, 20 min of static extraction time). The same set of samples was reanalyzed at a later date using the same optimized SFE conditions. S_0 was calculated from the linear regression of the standard deviation of the replicates versus the mean concentration of each level. The mean concentrations of the samples were 16.345, 34.468, and 58.702 ppm, and the corresponding standard deviations for (n = 6) six replicate analyses at each concentration level were 0.783, 1.517, and 1.342, respectively. An S_0 of 0.774 was obtained from the linear regression of the standard deviation of the replicates versus concentration. The MDL was then calculated as ($3 \times S_0$) 2.320 ppm. However, a lower detection limit of 0.15 ppm was calculated as 3 times the standard deviation of the measurements at the lowest measurable concentration; the limit was within the practically measurable standard FB_1 concentrations.

CONCLUSION

SFE provides a fast, effective, and reproducible method for the extraction of fumonisin from grain and grain dust samples. The method is suited for occupational or environmental exposure measurement since it has been optimized for small samples (<0.25 g) that are difficult to extract by the liquid−liquid extraction method. The SFE method reduces the use and exposure to organic solvents. The 40× magnitude of increased recovery of FB_1 from grain matrix samples demonstrates the efficacy of SC-CO_2 to extract fumonisin B_1 from solid matrices. The SFE is also compatible with on-line HPLC/MS analysis, which is part of our current effort to enhance the sensitivity for the analysis of mycotoxins in small amounts (<100 mg) of grain dust.

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