Published online April 3, 2006

Determination of Steroidal Estrogens in Flushed Dairy Manure Wastewater by Gas Chromatography–Mass Spectrometry

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ABSTRACT

There is a critical need to accurately measure the concentrations of natural steroidal estrogens in flushed dairy manure wastewater (FDMW) to assess any potential risk of waterway contamination resulting from land application. Estrogens are a concern because low concentrations (10–100 ng L^{-1}) in water can adversely affect aquatic vertebrate species such as fish, turtles, and frogs by disrupting the normal function of their endocrine systems. The objective of this study was to develop a sample preparation method that permits the quantification of four natural steroidal estrogens (17α-estradiol, 17β-estradiol, estrone, and estriol) in FDMW by gas chromatography–mass spectrometry (GC–MS). Solid-phase extraction with graphitized carbon black was used for the bulk extraction of estrogens from FDMW and additional sample purification was accomplished with C-18. The sample preparation method allowed estrogens to be detected accurately by GC–MS in FDMW. Spiked recovery experiments indicated that the method is satisfactory for measuring the estrogens of interest in FDMW with average recovery of >90%. As expected in FDMW, characterization of the estrogen profile revealed a large abundance of 17α-estradiol relative to 17β-estradiol and estrone. Estriol was not detected in FDMW. The methodology developed in this research helps provide an analytical foundation for the quantification of steroidal estrogens in FDMW by GC–MS.

Livestock manure contains appreciable amounts of natural steroidal estrogen hormones, such as estradiol, estrone, and estriol, that can potentially contaminate surface and ground water (Shore et al., 1993; Finlay-Moore et al., 2000; Hanselman et al., 2003; Raman et al., 2004). Estrogen contamination of water resources is a concern because low part per trillion concentrations (10–100 ng L^{-1}) of these chemicals may adversely affect the reproductive biology of aquatic vertebrates by disrupting the normal function of their endocrine systems (Jobling et al., 1998; Tyler et al., 1998; Panter et al., 1998; Irwin et al., 2001).

The ecological hazard, if any, posed by steroidal estrogens resulting from dairy production is not clearly known. Nevertheless, based on the amount of estrogens excreted in urine and feces, Lange et al. (2002) estimated that pregnant and cycling cows (*Bos taurus*) are responsible for about 90% of the steroidal estrogen input to the environment by domestic livestock in the United States and Europe. Therefore, it is critically important to know the types and amounts of steroidal estrogens that occur in dairy wastes so that endocrine disruption risks can be minimized or avoided.

Freshly voided dairy manure is a mixture of undigested dietary residues, gut microflora, and their metabolic end-products. As collected, the manure may include spilled feed, bedding materials, and water, in addition to feces and urine. Typically, manure is either scraped or flushed from the barns (Wilkie, 2005). Many dairies use hydraulic flushing for manure management, followed by primary treatment (mechanical screening or sedimentation, or both) to remove coarse solids. The liquid fraction of flushed dairy manure after settleable solids are removed is referred to as flushed dairy manure wastewater (FDMW) (Wilkie et al., 2004). Gauging the steroidal estrogen profile of FDMW or other livestock waste is not a trivial task, however, due to the low concentrations that must be measured, the difficulties associated with extracting estrogens from the waste, the chemical complexity of the resulting extract matrix, and the potential for degradation losses to occur during sample storage (Raman et al., 2001).

Quantitative enzyme immunoassays have been used in previous studies for measuring estrogens in dairy waste (Raman et al., 2001). However, previous work in our laboratory showed that enzyme immunoassays were prone to matrix interference which resulted in inaccurate quantification of estrogen in dairy waste (Hanselman et al., 2004). Gas chromatography–mass spectrometry (GC–MS) or liquid chromatography–mass spectrometry (LC–MS) based methods are more conclusive than immunoassays, but few of these techniques have been developed for measuring estrogens in livestock waste (Raman et al., 2001; Fine et al., 2003). Raman et al. (2001) developed a procedure for measuring 17α-estradiol, 17β-estradiol, and estrone in dairy waste by GC–MS. The sample preparation involved liquid–liquid extraction of estrogens from the waste sample using diethyl ether followed by BSTFA [*N*,*O*-bis(trimethylsilyl)fluoroacetamide] derivatization in DMF (dimethylformamide) (Raman et al., 2001). However, the detection limits reported by Raman et al. (2001) are poor (10 μg L^{-1}) relative to the endogenous concentrations of steroidal estrogens (ng L^{-1}) likely to be found in dilute dairy wastes such as FDMW (Kolodziej et al., 2004). Regardless of detection limit restrictions, liquid–liquid ether extraction of FDMW gives a sample that is not of sufficient quality for quantitative derivatization and introduction into the GC–MS (Hanselman et al., 2004). Thus, a more extensive extraction and purification technique is required for the analysis of wastes such as FDMW. The

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Published in J. Environ. Qual. 35:695–700 (2006).
Short Communications
doi:10.2134/jeq2005.0282

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Abbreviations: FDMW, flushed dairy manure wastewater; GC–MS, gas chromatography–mass spectrometry; SPE, solid-phase extraction.

objective of this study was to develop a sensitive and reliable sample preparation method that permits the quantification of four natural steroidal estrogens (17α-estradiol, 17β-estradiol, estrone, and estriol) in FDMW by GC–MS.

MATERIALS AND METHODS

Chemicals and Reagents

Estrone, 17α-estradiol, 17β-estradiol, estriol, and *N*,*N*-dimethylformamide (high performance liquid chromatography [HPLC]-grade), were purchased from Sigma-Aldrich (St. Louis, MO). Methanol (HPLC-grade), methylene chloride (HPLC-grade), acetone (Optima-grade), water (HPLC-grade), ethyl acetate (HPLC-grade), acetonitrile (Optima-grade), hexane (Optima-grade), and formic acid (ACS-grade) were purchased from Fisher Scientific (Hampton, NH). The derivatizing reagent, BSTFA, was purchased from Supelco (Bellefonte, PA). Internal standard mixture US-108 containing d_{12}–pyrelene was purchased from Ultra Scientific (Kingston, RI). Sample reservoirs (75 mL), filtration frits (approximately 20 μm), 500 mg Carbograph (graphitized carbon) solid-phase extraction (SPE) columns, 1000 mg C-18 (octadecylsiloxane-bonded silica) high-flow SPE columns, and nylon syringe filters (13 mm, 0.2 μm) were purchased from Alltech Associates (Deerfield, IL). Accubond II C-18 SPE cartridges (500 mg) were purchased from Agilent Technologies (Wilmington, DE).

Sample Collection

A single 1-L grab sample of fresh FDMW was collected on each of five consecutive days (19–23 Jan. 2004) from the University of Florida Dairy Research Unit located at Hague, FL, transported on ice within 1 h to the laboratory in Gainesville, FL, and immediately extracted. The total solids content of the FDMW sample collected each day was determined by standard methods (American Public Health Association, 1998) to be 0.79, 1.04, 0.66, 1.31, and 0.91%, respectively.

Initial Preparation of Samples and Matrix Spikes

From each of the five bulk daily samples (designated as FDMW 1 through 5), eight replicate aliquots (40 mL each) were measured into separate 50-mL Teflon tubes and centrifuged in an Eppendorf 5810 (Brinkmann Instruments, Westbury, NY) at 15 000 × *g* for 15 min to pelletize suspended solids. Each clarified supernatant was transferred into a 125-mL flask without disturbing the pellet and set aside. Estrogens adsorbed to pelletized solids were extracted with 10 mL methanol in a 40°C ultrasonic bath for 30 min. After centrifugation at 4000 × *g* for 15 min, the methanol extract was combined with the aqueous portion of the sample and set aside. The pellet was extracted once more with 10 mL of methanol for 30 min in a 40°C ultrasonic bath and, after centrifuging at 4000 × *g* for 15 min, the methanol extract was added to the previous supernatant and mixed thoroughly.

At this point, four replicates of each sample were selected to continue processing as samples. To monitor the efficiency of the solid-phase extraction series when applied to the complex matrix left from bulk-extracted FDMW samples, the remaining replicates were fortified with known amounts (40 ng each) of 17α-estradiol, 17β-estradiol, estrone, and estriol from a 1000 ng mL^{-1} stock solution in acetone. Samples were spiked after centrifugation and methanol extraction to minimize microbial degradation of the target analytes. All spiked samples were prepared and analyzed with their corresponding unspiked sample replicates.

Extraction efficiency was calculated by dividing the measured concentration of estrogens in the spiked sample by the theoretically expected concentration in spiked samples and the result was multiplied by 100. To assess the extraction efficiency at different levels, an additional set ($n = 4$) of sample replicates were prepared from the FDMW 5 sample for each of four spiking amounts (20, 40, 60, and 80 ng each of 17α-estradiol, 17β-estradiol, estrone, and estriol).

Bulk Solid-Phase Extraction

Estrogens were extracted from the replicate samples using Carbograph solid-phase extraction (Andreolini et al., 1987; Baronti et al., 2000; Lagana et al., 2000, 2001; Gentili et al., 2002). The samples were poured into fritted reservoirs and passed through Carbograph SPE columns sequentially preconditioned with 10 mL methylene chloride–methanol (80:20 v/v), 5 mL methanol, and 10 mL water acidified to pH 2. The samples were passed through the columns with the aid of vacuum at 5 to 10 mL min^{-1}. Once the sample passed, the flasks were rinsed with 50 mL of water and the rinse was applied to the columns. After rinsing, the reservoir was removed and the Carbograph column was washed sequentially with 5 mL of 75% methanol acidified with 100 mmol L^{-1} formic acid and 5 mL of 75% methanol. The base/neutral fraction of retained organics that included the target estrogens was eluted with 2 mL methanol and 15 mL of methylene chloride–methanol (80:20 v/v) into 50-mL flasks. The captured eluant was heated at 70°C under a gentle stream of N_2 until the methylene chloride evaporated. After cooling, 50 mL of HPLC-grade water was added to the residual methanol and mixed by swirling.

Sample Purification and Derivatization

To improve sample purity, C-18 SPE was performed. The aqueous-solvent sample mixtures resulting from Carbograph extraction were poured into reservoirs and passed with the aid of vacuum at 5 to 10 mL min^{-1} through C-18 columns preconditioned with 5 mL acetone and 5 mL water. After the samples passed, the flasks were rinsed with 50 mL of water and the rinse was applied to the C-18 column. When the rinse passed through, vacuum was applied to the columns for an additional approximately 15 min to remove excess water. A nylon syringe filter was attached to the bottom of each C-18 column and estrogens were eluted with 4 mL of acetone. Sample volumes were adjusted to a final volume of 4.0 mL in acetone, capped tightly, and stored at −20°C.

In preparation for GC–MS analysis, an additional SPE step was required to reduce matrix background. The samples were taken to dryness under nitrogen. After resuspending each residue in 0.2 mL of methanol with vortexing, 4 mL of water was added to each vial. The samples were then applied to 500 mg C-18 cartridges (preconditioned sequentially with 4 mL methanol and 4 mL water) and allowed to percolate through the columns by gravity. The cartridges were then washed with 4 mL of water, dried under vacuum for 3 min, washed with 4 mL of hexane, and eluted with two 4-mL portions of ethyl acetate. The resulting extracts were taken to dryness under nitrogen in preparation for derivatization. To each vial, 400 μL of DMF and 100 μL of BSTFA were added, after which the solutions were vortexed, capped, and allowed to incubate for 16 h at room temperature. The derivatized products were then taken to dryness under nitrogen, redissolved in 500 μL of acetonitrile, spiked with internal standard d_{12}–pyrelene (from US-108) to a final concentration of 1 μg mL^{-1}, and analyzed.

Gas Chromatography–Mass Spectrometry Analysis

Estrogens were separated using an HP-6890 gas chromatograph (Hewlett-Packard, Palo Alto, CA) with split/splitless inlet operated in splitless mode at 260°C. The sample was introduced in a 1-μL injection and separated across an HP-5MS column (30 m × 0.25 mm; 0.25-μm film thickness; J & W Scientific, Folsom, CA) under a temperature program that began at 200°C, held for 2 min, then increased at 10°C min^{-1} to 300°C with a 10-min hold. The He flow rate was 1 mL min^{-1}. Estrogen detection was accomplished using an HP 5973 mass selective detector operated in electron impact mode with a source temperature of 230°C and a quadrupole temperature of 150°C. Identification of all analytes was conducted in full scan mode, where all ions are monitored. For improved sensitivity, selected ion monitoring was used for quantitation. The ions monitored for 17α-estradiol and 17β-estradiol were m/z = 285 and 416, for estriol, m/z = 504 and 345, and for estrone, m/z = 342 and 257.

For quantitation, a standard curve containing at least six points was prepared for each analyte ($R^2 \geq 0.995$). Calibration standards were prepared in acetonitrile and fresh curves were analyzed with each set of samples. Like each sample, each standard was fortified to contain deuterated internal standard, d_{12}–pyrelene, at a final concentration of 1 μg mL^{-1}. Standard curves ranged from 10 pg μL^{-1} to 400 pg μL^{-1} with $R^2 \geq 0.995$ for all analytes but estriol, which ranged from 20 pg μL^{-1} to 400 pg μL^{-1} with $R^2 \geq 0.995$. The calibration curves for all analytes were consistent throughout the course of the study. Water blanks, prepared and analyzed alongside standards and samples, were found to contain no detectable levels of any analyte. Method detection limits were based on the lowest concentrations of the analytes that could be accurately and repeatedly identified against the matrix background. The values were estimated against low level standards. Quantitation limits were based on the lowest concentrations of the analytes that could be accurately and precisely quantified from the matrix. At the lowest standard on the calibration curve, RSD precision was ≤20%.

RESULTS AND DISCUSSION

Extraction Method Performance

Spiked recovery of 40 ng 17α-estradiol, 17β-estradiol, estrone, and estriol averaged 96, 125, 101, and 99%, respectively (n = 4 for each of five samples; Table 1). Method precision (RSD ≤ 12%) was also acceptable for all target analytes (Table 1). As shown in Fig. 1, the net amount of each estrogen extracted from FDMW after spiking with 20, 40, 60, and 80 ng was linear within the range evaluated. Recovery of the four spiking levels of 17α-estradiol, 17β-estradiol, estrone, and estriol averaged 100, 122, 99, and 92%, respectively (n = 4). Precision (RSD ≤ 12%) was also acceptable for each analyte at the four spiking levels evaluated.

Table 1. Percentage recovery of spiked estrogens from five samples of flushed dairy manure wastewater.

FDMW†	Estrone	17α-Estradiol	17β-Estradiol	Estriol
		%		
1	92 (5)‡	96 (6)	116 (5)	90 (9)
2	104 (5)	105 (5)	134 (8)	99 (9)
3	105 (2)	93 (5)	121 (2)	109 (5)
4	107 (7)	94 (10)	139 (8)	107 (10)
5	98 (7)	94 (9)	114 (8)	90 (12)
Average	101 (5)	96 (7)	125 (6)	99 (9)

† Flushed dairy manure wastewater.
‡ Mean values with (RSD), n = 4.

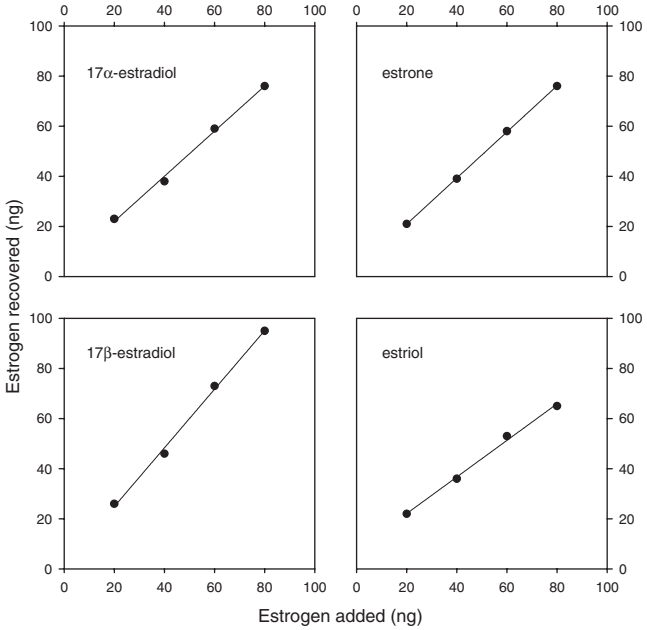

Fig. 1. Net amount of 17α-estradiol, estrone, 17β-estradiol, and estriol extracted from flushed dairy manure wastewater (FDMW) (n = 4) after spiking with 20, 40, 60, and 80 ng of target analytes.

Overall, the spiked recovery experiment demonstrates that Carbograph SPE and C-18 purification is a reliable sample preparation method for the sensitive determination of estrogens in FDMW by GC–MS. The Carbograph/C-18 extraction and purification method used in this study compares favorably with other research involving SPE of estrogens from environmental matrices. For example, Baronti et al. (2000) reported ≥86% recovery of added 17β-estradiol, estrone, and estriol from aerobic sewage treatment plant influent and effluent, and river water when using Carbograph SPE, and Lee and Peart (1998) reported ≥98% recovery of added 17β-estradiol, estrone, and estriol from sewage effluent by C-18 SPE.

Gas Chromatography–Mass Spectrometry Performance

Using the methodology developed in this study, the limit of detection for each analyte was determined to be 75 ng L^{-1} FDMW with a limit of quantitation of 125 ng L^{-1} for 17α-estradiol, 17β-estradiol, and estrone, and 250 ng L^{-1} for estriol. These parameters represent a significant improvement in sensitivity for the analysis of dairy waste samples as compared with the method of Raman et al. (2001) who reported a GC–MS detection limit of approximately 10 000 ng L^{-1}. Furthermore, our work confirmed that direct injection of the DMF/BSTFA derivative as specified in their paper resulted in the rapid and severe degradation of the GC column (Raman et al., 2001). Drying and resuspending the

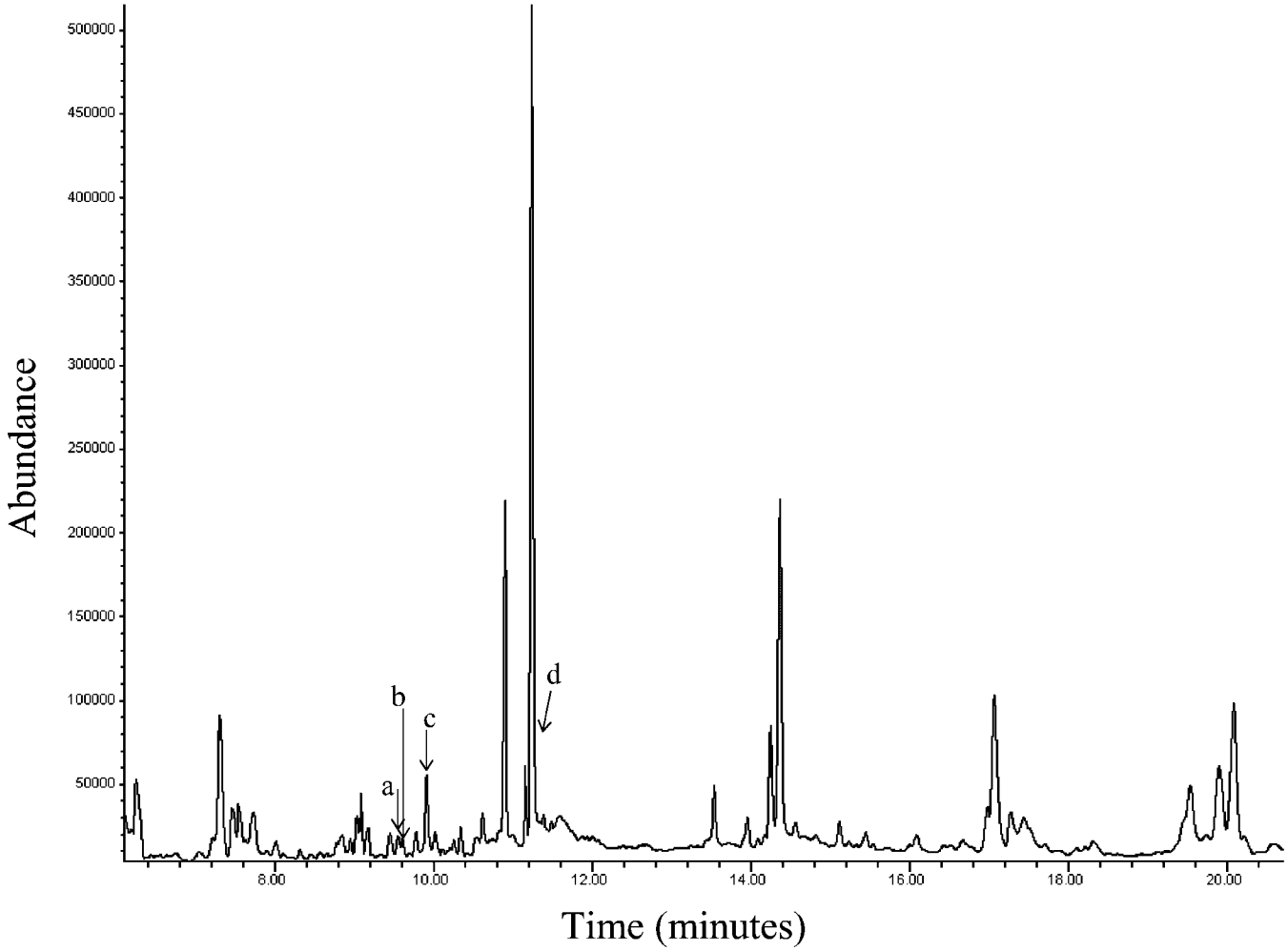

Fig. 2. Selected ion chromatogram of unspiked flushed dairy manure wastewater (FDMW) showing the retention time of the BSTFA [*N,O*-bis(trimethylsilyl)fluoroacetamide] derivatives of (a) estrone, (b) 17α-estradiol, (c) 17β-estradiol, and the internal standard (d) d_{12}–pyrelene.

derivatized product in acetonitrile was found to alleviate column degradation which permitted the analysis of all study samples on the same GC column with minimal instrument maintenance. Selected ion chromatograms for an unspiked and a corresponding 40-ng spiked FDMW replicate sample are shown in Fig. 2 and 3, respectively. The chromatograms illustrate the typical retention times of the BSTFA estrogen derivatives and the typical matrix background observed with FDMW samples using the proposed sample preparation methodology.

Estrogen Concentrations in Flushed Dairy Manure Wastewater

The endogenous concentration of estrogens in five samples of FDMW determined by GC–MS is shown in Table 2. Estrone, 17α-estradiol, and 17β-estradiol concentrations averaged 879, 2282, and 643 ng L^{-1}, respectively, but estriol was not detected during five consecutive sampling days. The absence of estriol and the abundance of 17α-estradiol relative to 17β-estradiol and estrone is consistent with the estrogen excretion profile of cattle (Mellin et al., 1965; Hoffmann et al., 1997).

It is difficult to compare in a meaningful way the estrogen concentrations in FDMW with other types of dairy manure samples because FDMW is highly dilute due to extensive flushwater volumes. Herd size and storage duration also affect the measured estrogen concentrations significantly. However, compared with other low solids content dairy wastes such as from holding ponds, estrogen concentrations in FDMW appear to be less (Williams, 2002; Raman et al., 2004). For example, Williams (2002) reported GC–MS measured concentrations of estrone, 17α-estradiol, and 17β-estradiol in dairy holding ponds from scraped dairy operations averaged 7595, 5185, and 3350 ng L^{-1}, respectively. However, as mentioned previously, the detection limits associated with the sample preparation frequently hindered GC–MS quantification of estrogens and resulted in a high frequency of "below detectable limits" reported for several dairy waste samples (Williams, 2002). For example, 87 and 60% of samples collected from dairy holding ponds were below the method detection limits for 17β-estradiol and estrone, respectively (Williams, 2002). The

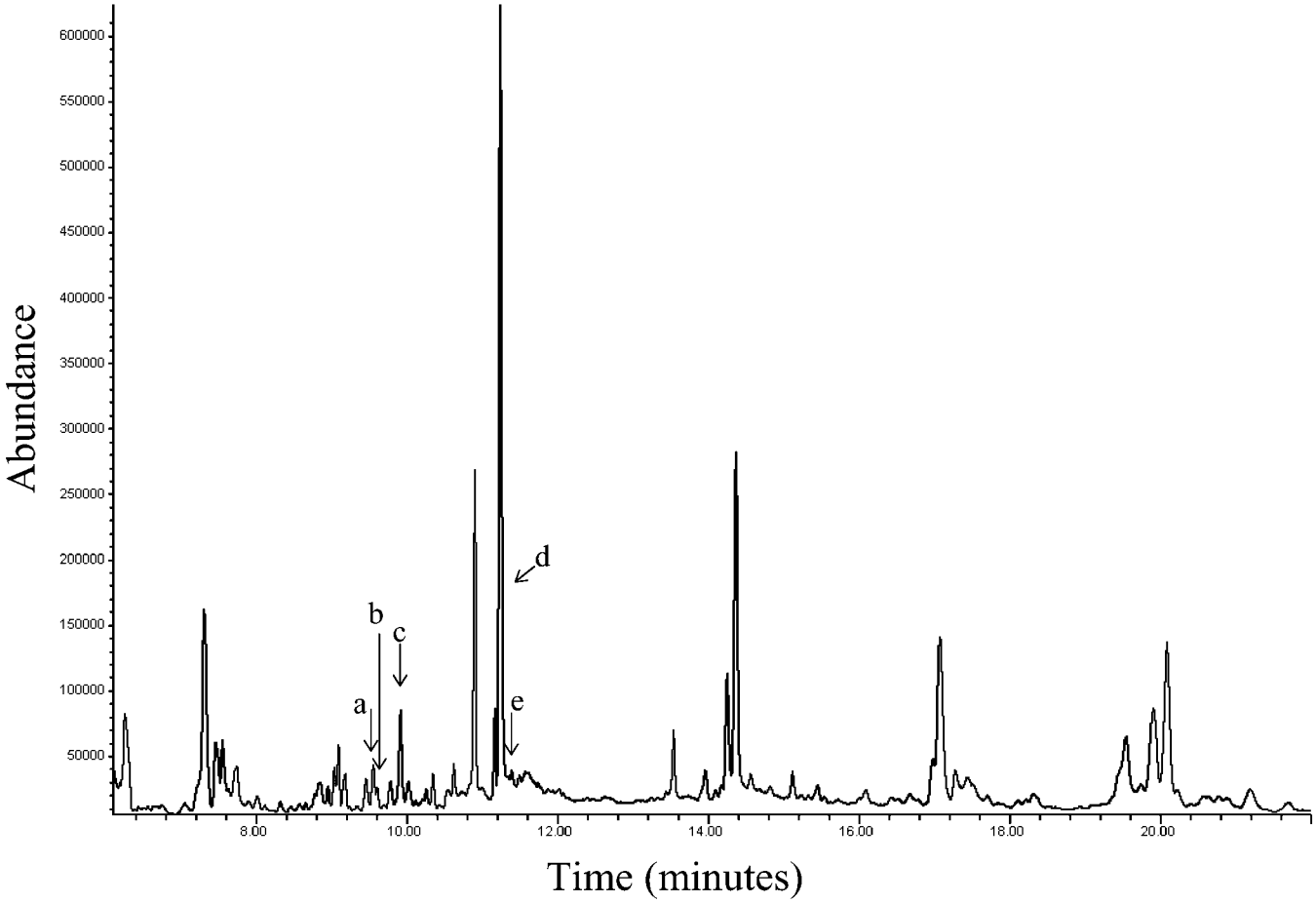

Fig. 3. Selected ion chromatogram of spiked flushed dairy manure wastewater (FDMW) showing the retention time of the BSTFA [*N,O*-bis(trimethylsilyl)fluoroacetamide] derivatives of (a) estrone, (b) 17α-estradiol, (c) 17β-estradiol, the internal standard (d) d_{12}–pyrelene, and (e) estriol after spiking with 40 ng of each estrogen analyte.

estrone concentrations reported in the present study appear to be consistent with concentrations reported by Kolodziej et al. (2004) for FDMW using GC–MS–MS technology. They found estrone concentrations up to 650 ng L^{-1} in a dairy waste lagoon from a flushed dairy operation (Kolodziej et al., 2004). 17β-Estradiol was not detected, which may be a result of degradation during storage. Unfortunately, 17α-estradiol—the primary endogenous estrogen excreted by cattle (Mellin and Erb, 1966; Hoffmann et al., 1997)—concentrations of the manure cannot be compared since they were not measured in their study.

Table 2. Estrogen concentrations in five samples of flushed dairy manure wastewater measured by gas chromatography–mass spectrometry (GC–MS).

FDMW†	Estrone	17α-Estradiol	17β-Estradiol	Estriol
	ng L^{-1}			
1	2356 ± 74‡	2036 ± 92	711 ± 52	BDL§
2	467 ± 66	1750 ± 62	525 ± 42	BDL
3	650 ± 22	3270 ± 99	957 ± 22	BDL
4	370 ± 46	2114 ± 98	351 ± 17	BDL
5	551 ± 50	2239 ± 160	672 ± 32	BDL

† Flushed dairy manure wastewater.
‡ Mean values ± SE, $n = 4$.
§ Below detectable limits.

CONCLUSIONS

A new sample preparation method involving liquid and solid-phase extraction was developed for the measurement of 17α-estradiol, 17β-estradiol, estrone, and estriol in FDMW by GC–MS. Results of spiking experiments indicated that the method is satisfactory for determining four estrogens in FDMW with recovery of >90%. Characterization of the estrogen profile of FDMW revealed a large abundance of 17α-estradiol relative to 17β-estradiol and estrone. Estriol was not detected in FDMW. The methodology developed in this research appears useful for the analysis of steroidal estrogens in dairy wastewater and provides some analytical foundation for future research involving the determination of estrogens in livestock wastes.

ACKNOWLEDGMENTS

This research was supported by the University of Florida School of Natural Resources and Environment Mini-Grants Program.

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