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SHORT COMMUNICATIONS

Comparison of Three Enzyme Immunoassays for Measuring 17β-Estradiol in Flushed Dairy Manure Wastewater

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Abstract

Natural steroidal estrogens are an environmental concern because low nanogram per liter concentrations in water can adversely affect aquatic vertebrate species by disrupting the normal function of their endocrine systems. There is a critical need to accurately measure estrogens in dairy wastes, a potential source of estrogens such as 17β-estradiol, to assess the risk of estrogen contamination of agricultural drainage waters resulting from land application. Commercially available enzyme immunoassay (EIA) kits have been used for measuring 17β-estradiol in livestock manure, but it is not known if different EIAs provide similar results. We compared three EIAs by measuring 17β-estradiol in two samples of flushed dairy manure wastewater (FDMW). The measured concentrations of 17β-estradiol in FDMW differed according to the immunoassay used. The differences were attributed to a matrix interference associated with coextracted humic substances. Future research should develop methods that enable routine measurement of 17β-estradiol in livestock wastes by more conclusive analytical techniques such as gas chromatography–mass spectrometry.

Dairy farms in the United States generate approximately 21.5 million Mg of recoverable manure solids each year that must be managed in a way that does not adversely impact the environment (USEPA, 2001). Typically, dairy wastes are applied to nearby pasture and croplands as soil amendments because they contain various plant nutrients, including N, P, and K. However, agricultural drainage waters may become contaminated with natural steroidal estrogen hormones such as 17β-estradiol when livestock wastes are land-applied (Shore et al., 1995; Nichols et al., 1997, 1998; Bushee et al., 1998; Finlay-Moore et al., 2000; Dyer et al., 2001).

Estrogen contamination of waterways is a concern because low concentrations (10–100 ng L^{-1}) of these chemicals in water can adversely affect the reproductive biology of vertebrate species such as fish, turtles, and frogs by disrupting the normal function of their endocrine systems (Panter et al., 1998, 2000; Tyler et al., 1998; Irwin et al., 2001; Oberdorster and Cheek, 2001). For example, 17β-estradiol concentrations of $\geq$30 ng L^{-1} induced vitellogenin (an egg yolk precursor protein that is normally produced only by adult females) synthesis and abnormal testicular growth in male fathead minnows (*Pimephales promelas*) after 21 d of laboratory exposure (Panter et al., 2000). However, research evaluating the in situ effects of manure-borne estrogens on wildlife is limited. Irwin et al. (2001) reported that vitellogenin production by female painted turtles (*Chrysemys picta*) in ponds was significantly affected by estrogens in beef cattle runoff compared with turtles in ponds unexposed to beef cattle runoff.

Clearly, it is important to have accurate information about the occurrence of estrogens in manure so that any estrogen contamination of waterways resulting from dairy waste disposal can be prevented or minimized. Estrogen characterization of dairy wastes is not a trivial task, however, due to the low concentrations that must be measured, the difficulties associated with extracting estrogens from manure, the chemical complexity of the resulting extract matrix, and the potential for degradation losses to occur during sample storage (Raman et al., 2001). A variety of quantitative EIAs have been used for the determination of 17β-estradiol in manure-impacted surface and ground water and in livestock wastes (Nichols et al., 1997; Bushee et al., 1998; Peterson et al., 2000; Finlay-Moore et al., 2000). The popularity of EIA for estradiol analysis is attributable to widespread commercial availability, ease of use, pg mL^{-1} detection limits, and a lack of alternative quantitation methods. However, a variety of interferences, arising from poor standardization, cross-reactivity, and matrix effects associated with protein binding, humic substances, and endogenous enzymes, can adversely affect the quality (accuracy, precision, reproducibility) of the data produced (Wood, 1991; Maxey et al., 1992; Nunes et al., 1998; Huang and Sedlak, 2001). Thus, depending on sample complexity and EIA reagents, antibodies, and protocol, a potential exists for different EIA systems to yield dissimilar and/or inaccurate results. The objective of this study was to determine if three different commercially available 17β-estradiol EIAs yielded similar estimates of the endogenous concentration of 17β-estradiol in flushed dairy manure wastewater.

Materials and Methods

Sample Collection

Many dairies use hydraulic flushing for manure management, followed by primary treatment (mechanical screening

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Abbreviations: EIA, enzyme immunoassay; FDMW, flushed dairy manure wastewater; GC–MS, gas chromatography–mass spectrometry.

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or sedimentation, or both) to remove coarse solids. The liquid fraction of flushed dairy manure after settleable solids are removed is referred to as FDMW (Wilkie et al., 2004). A 1-L grab sample of FDMW was collected from the University of Florida Dairy Research Unit located at Hague, FL, and immediately (<1 h) transported to the laboratory for liquid–liquid ether extraction. Two weeks later, a second 1-L sample of FDMW was collected and processed in a similar manner. The total solids content of these samples was determined by a standard method (American Public Health Association, 1998). The first and second FDMW samples contained an average of 0.57 and 0.62% total solids, respectively.

Extraction

For each wastewater sample, four aliquots (20 mL) of FDMW were poured into separate 50-mL glass centrifuge tubes. Twenty milliliters of pesticide-grade ethyl ether (Fisher Scientific, Hampton, NH) was added to each tube for extraction of 17β-estradiol. Liquid–liquid extraction with ether was used for sample preparation because it is a traditional solvent of choice for steroid extraction from biological samples; ether extraction is recommended for sample purification by the EIA manufacturers used in this study, and it has been used previously for extraction and purification of dairy waste samples for EIA analysis (Raman et al., 2001).

The tubes were shaken horizontally for 2 h followed by centrifugation at 500 × *g* for 5 min to facilitate layer separation. Three 4-mL aliquots (one for each assay) of the ether extract were subsampled from each tube and placed into separate 5-mL evaporation flasks. The ether was evaporated to dryness at 40°C under N_2. The dried sample was immediately reconstituted in 1 mL of bulk assay buffer that was purchased from each immunoassay manufacturer. The reconstituted samples were individually sonicated for approximately 1 min to enhance solubilization in the assay buffer. The samples were poured into 1.5-mL micro-centrifuge tubes, capped tightly, and stored overnight (−20°C) before immunoassay analysis.

Immunoassay Description

Enzyme immunoassay kits for the quantitative determination of 17β-estradiol were purchased from Assay Designs (Catalog no. 900-008; Ann Arbor, MI), Diagnostics Systems Laboratories (Catalog no. DSL-10-4300; Webster, TX), and Immuno-Biological Laboratories (Catalog no. RE 52041; Minneapolis, MN). The immunoassay kits were designated A1, A2, and A3, respectively. The A1 immunoassay (Catalog no. 900-008) was selected because it has been used previously for the quantification of 17β-estradiol in dairy wastes (Raman et al., 2001). The A2 and A3 immunoassays were selected based on their use of rabbit polyclonal antibodies (RPA) and the competitive assay principle, and a low cross-reactivity with other steroids (Table 1).

Each of the EIAs used in this study were based on the competitive binding principle, whereby 17β-estradiol and a fixed amount of enzyme-labeled estradiol compete for RPA binding sites. However, the A2 and A3 assays use RPAs that are directly coated onto the microplate wells, whereas the A1 microplate wells are coated with goat anti-rabbit IgG to capture the 17β-estradiol–RPA complex. The alkaline phosphatase, streptavidin-horseradish peroxidase, and horseradish peroxidase enzyme tracers used by A1, A2, and A3, respectively, represent commonly used enzyme reagents for estrogen immunoassay (Table 1) (Meyer et al., 1990; DeBoever et al., 1995; Mares et al., 1995; Vos, 1996). As shown in Table 1, each immunoassay has a low (<5%) cross-reactivity with other estrogen steroids.

Immunoassay Analysis

Each assay was performed according to the manufacturer's instructions. All standards and samples were assayed in duplicate and an average value was used to generate standard curves and interpolate unknown sample concentrations. Microplate washing was performed with an $EL_x50/8$ strip washer (Bio-Tek Instruments, Winooski, VT) using the wash buffer reagents provided by each company. The absorbance values of each well were measured using an FL 600 microplate reader (Bio-Tek Instruments). A four-parameter logistic equation was used for all calibration curves (Rodbard and Lewald, 1974).

Immunoassay performance characteristics including sensitivity, standardization, precision, and recovery of diluted and spiked samples were evaluated on both days of wastewater analysis. Sensitivity is defined as the lowest measurable concentration of 17β-estradiol that can be distinguished from the respective 0 pg mL^{-1} calibrator (95% confidence interval) associated with each EIA (Vadlamudi et al., 1991). Sensitivity was calculated for each EIA by interpolation of the mean of eight replicate samples of the respective 0 pg mL^{-1} calibrator minus two standard deviations.

Standardization accuracy refers to the ability of each EIA to yield a correct measurement of 17β-estradiol for a known standard concentration. Standardization accuracy was evaluated at three concentrations (1500, 750, and 375 pg mL^{-1}) by diluting a 300 000 pg 17β-estradiol mL^{-1} buffer solution (Assay Designs) with the respective 0 pg mL^{-1} calibrator of each EIA. Three concentrations were measured to ensure accurate recov-

Table 1. Description and cross-reactivity of three enzyme immunoassay systems used for measuring 17β-estradiol in flushed dairy manure wastewater.†

Description	A1	A2	A3
Assay principle	competitive	competitive	competitive
17β-Estradiol antibody	rabbit polyclonal	rabbit polyclonal	rabbit polyclonal
Matrix	TBS	serum	serum
Conjugate, enzyme	E2-ALP	E2-biotin, SHRP	E2-HRP
Substrate	*p*-NPP	TMB	TMB
Range, pg mL^{-1}	0–30 000	0–6000	0–2000
MDL, pg mL^{-1}	29	7	10
Precision, % CV	9	4	4
Cross-reactivity, %			
17β-estradiol	100	100	100
17α-estradiol	0.1	0.3	0.3
Estrone	4.6	1.4	2.1
Estriol	0.5	1.1	1.5

† TBS, Tris-buffered saline containing proteins and detergents and sodium azide as a preservative; E2, 17β-estradiol; ALP, alkaline phosphatase; SHRP, streptavidin horseradish peroxidase; HRP, horseradish peroxidase; *p*-NPP, *p*-nitrophenol phosphate; TMB, tetramethylbenzidine; MDL, minimum detection limit.

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ery at different interpolation points along the calibration curve. A recovery percentage for each standard concentration was calculated by dividing the measured sample concentration by the known sample concentration and multiplying the result by 100. The three resulting values were averaged to express EIA standardization accuracy.

Intra-assay precision refers to the within-run reproducibility of the 17β-estradiol signal that is produced for a particular sample in an EIA. We evaluated precision by calculating the percent coefficient of variation observed between duplicate measurements corresponding to the four neat wastewater samples. The four resulting % CV values were averaged to express precision.

Recovery of diluted and spiked samples is a gauge of the linear relationship between 17β-estradiol measured in diluted or spiked samples relative to the neat samples. Dilution recovery was measured by diluting each of the four neat wastewater samples with an equal volume of the respective 0 pg mL^{-1} calibrator of each assay. Spiked recovery was measured by spiking the neat wastewater samples with an equal volume of the second greatest respective 17β-estradiol calibrator from each EIA (i.e., A1, 7500 pg mL^{-1}; A2, 2000 pg mL^{-1}; A3, 1000 pg mL^{-1}). The second greatest calibrators were used for spiking to ensure that the resulting spiked sample concentrations would be interpolated from the mid-portion of the calibration curve of each assay. Dilution and spiked recovery was expressed as a percentage by dividing the measured concentration of the diluted or spiked sample by the theoretically expected concentration of the diluted or spiked sample, and the result was multiplied by 100.

Data Analysis

The experimental design was a two-way factorial (three immunoassay methods × two FDMW samples) with four replications. Experimental data were analyzed using the General Linear Model program of SAS with a separation of sample means by Duncan's new multiple range test (SAS Institute, 2000).

Results and Discussion

A summary of the immunoassay performance characteristics from each FDMW analysis is shown in Table 2. The measured sensitivity data corresponding to the first wastewater sample were similar to or better than the manufacturer's data for each EIA. However, the sensitivity data corresponding to the second analysis were somewhat larger for each assay. The average EIA sensitivity for both analyses was 62, 14, and 26 pg mL^{-1} for the A1, A2, and A3 assays, respectively. The sensitivity data demonstrate the exceptionally low 17β-estradiol concentrations that can be measured using EIA.

Table 2. Summary of performance data for analysis of two flushed dairy manure wastewater samples by three different immunoassays.

Performance characteristic	FDMW†	*n*	A1	A2	A3
Sensitivity, pg mL^{-1}	1	8	25	7	10
	2	8	98	20	41
Standardization accuracy, %	1	3	102	88	136
	2	3	108	108	75
Precision of replicate samples, % CV	1	4	13	9	11
	2	4	3	4	7
Recovery of diluted samples, %	1	4	92	109	124
	2	4	66	128	124
Recovery of spiked samples, %	1	4	88	101	96
	2	4	96	89	85

† Flushed dairy manure wastewater.

Recovery data shown in Table 2 demonstrate that the A1 and A2 assays were relatively well standardized for both analyses. The calibration of the A3 assay appeared to be somewhat less accurate for each individual analysis since it overestimated by 36% and underestimated by 25%, respectively, the standard concentrations for the first and second analysis. Overall, however, the average recovery percentage for both analyses was 105, 98, and 106% for the A1, A2, and A3 immunoassays, respectively. Therefore, it seems that each of the EIAs was reasonably well standardized.

Each assay also showed a high degree of intra-assay precision between duplicate samples. The % CV for both analyses averaged 8, 7, and 9%, respectively, for the A1, A2, and A3 assays. The low % CV values indicate that the chemical reactions involved in generating the 17β-estradiol signals for each EIA was highly reproducible within the analytical run.

The recovery of diluted samples ranged from 66 to 128%, depending on the EIA and day of analysis (Table 2). The recovery of diluted samples for both analyses averaged 79, 119, and 124%, respectively, for the A1, A2, and A3 assays. In contrast to diluted samples, recovery improved markedly when the neat samples were spiked with 17β-estradiol. The recovery of the spiked samples averaged 92, 95, and 91%, respectively, for the A1, A2, and A3 immunoassays. Overall, the recovery of diluted and spiked samples demonstrates a reasonably linear recovery of 17β-estradiol at the different interpolation points evaluated from the standard curve.

Although some minor differences were encountered between assays regarding standardization accuracy, intra-assay precision, and recovery of diluted and spiked samples, the measured concentration of 17β-estradiol in both sets of FDMW samples differed according to the EIA used (Fig. 1). The A1 assay consistently measured the greatest 17β-estradiol concentrations and the A2 assay measured the lowest. The average concentration of 17β-estradiol in the first wastewater sample measured with the A1, A2, and A3 immunoassays was 526, 161, and 332 ng L^{-1}, respectively, and 1310, 181, and 356 ng L^{-1}, respectively, in the second wastewater sample.

Because no differences were observed between EIAs when a pure solution of 17β-estradiol was analyzed (standardization accuracy) (Table 2), the apparent difference between assays suggests that an interference affected 17β-estradiol quantitation in FDMW samples in one or more of the EIAs. A known source of interference with the EIAs is the presence of other steroidal estrogens that are listed as cross-reactants in Table 1. It was noticed that the apparent concentrations of 17β-estradiol in the wastewater followed in the same qualitative order (A1 > A3 > A2) as the reported estrone cross-reactivity of the different assays. Consequently, estrone was a suspected source of bias between assays. Hence, we measured estrone with an estrone EIA (Catalog no. DB 520 51; Immuno-Biological Laboratories). Similar estrone EIAs were not available from the other companies for comparison.

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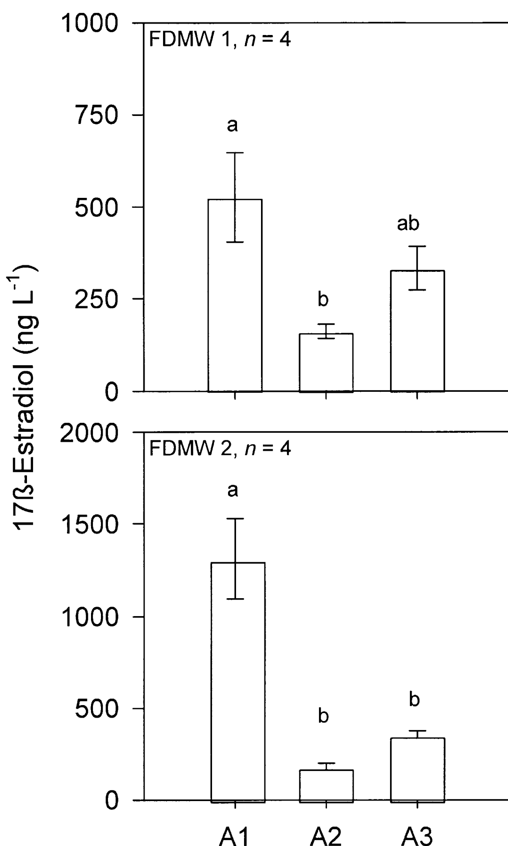

Fig. 1. Apparent concentration of 17β-estradiol in flushed dairy manure wastewater (FDMW) samples measured by three immunoassays. Different letters (a,b) indicate a significant difference ($\alpha = 0.05$) between sample means. Error bars denote standard error of the mean.

Estrone concentrations were 562 and 781 ng L^{-1} in the first and second wastewater samples, respectively. Based on the cross-reactivity data shown in Table 1, estrone in the first wastewater sample would have contributed approximately 26, 8, and 12 ng L^{-1} of 17β-estradiol signal to the A1, A2, and A3 assays, respectively. Likewise, estrone in the second set of wastewater samples would have contributed approximately 36, 11, and 16 ng L^{-1} to the 17β-estradiol signal. If the estrone cross-reactivity data provided by the manufacturers are correct and the EIA measured estrone concentrations are accurate, the large differences observed between assays do not appear to be caused by estrone cross-reactivity.

Other types of matrix interferences that are known to affect the quality of EIA data are often associated with coextracted humic substances. For example, Huang and Sedlak (2001) demonstrated that certain types of humic substances extracted from surface water could give positive signals during 17β-estradiol EIA. Presumably, the humic substances cross-react with the 17β-estradiol antibody or adsorb to the estradiol enzyme conjugate in a manner that inhibits the competitive antibody binding and thus give a false-positive EIA signal. On the other hand, humic substances may cause false-negative EIA signals if they inhibit the competitive binding of 17β-estradiol to the antibody binding sites.

Ideally, the lack of agreement between immunoassays could be reconciled with a more conclusive measurement technique like gas chromatography–mass spectrometry (GC–MS) to determine which assay provided the most accurate measurement of 17β-estradiol in FDMW. Unfortunately, GC–MS quantification was not possible with these wastewater samples due to the extraordinary sample complexity associated with the ether extracts and because the ng L^{-1} sample concentrations are several orders of magnitude lower than the detection limits (approximately 10 μg L^{-1}) associated with the only published method for the GC–MS analysis of dairy wastes (Raman et al., 2001). A similar problem was reported by Raman et al. (2001), who tried to compare the endogenous concentration of 17β-estradiol in press-cake dairy solids measured by the A1 EIA and GC–MS. Endogenous 17β-estradiol could not be measured by GC–MS due to the relatively poor detection limits. However, when 17β-estradiol was spiked into the press-cake samples, the A1 EIA and GC–MS methods agreed well. Nevertheless, the spiked EIA and GC–MS comparison does not yield much information regarding bias of the A1 assay because an interference, if present, would have been greatly masked by dilution of the spiked samples.

Based on the large differences observed between EIAs in this study, caution should be observed when interpreting the biological significance or ecological risk of 17β-estradiol concentrations in livestock wastes when measured by EIA. Immunoassays are potentially valuable tools for the rapid screening of environmental samples. However, a better understanding of the artifacts and interferences associated with highly complex and variable livestock waste matrices is clearly needed. To better understand EIA limitations, it is critical that sensitive and reliable GC–MS or liquid chromatography–mass spectrometry (LC–MS)-based methods be developed as definitive reference methods.

Conclusions

Ether extraction and quantitation by EIA is a convenient method for measuring estrogens in FDMW. Although no differences were observed between EIAs when a pure solution of 17β-estradiol was analyzed, three EIAs gave different 17β-estradiol results for the same wastewater samples. The differences are most likely caused by one or more matrix interferences associated with coextracted humic substances in the sample. The poor quality of the ether extracts and low concentrations of 17β-estradiol in the wastewater prevented GC–MS quantitation and therefore it is not known which of the three EIAs yielded the most accurate measurement of 17β-estradiol. Future research needs to develop better extraction and/or purification techniques so that 17β-estradiol and other estrogens can be measured in FDMW by more conclusive techniques like GC–MS or LC–MS and to ensure that immunoassay results can be validated.

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