Degradation of Estrogens in Dairy Waste Solids: Effects of Acidification and Temperature

D. Raj Raman
Alice C. Layton
Lara B. Moody
James P. Easter
Gary S. Sayler, et al.
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ABSTRACT. Manure–borne estrogens are increasingly recognized as a potential ecological hazard. However, sample–handling protocols for these compounds are not clearly delineated in the literature, nor are comparisons between assays for estrogens. A study was conducted to explore the degradation of estrogen in separated dairy manure waste solids (press cake), using three popular assay types. Estrogens were measured by enzyme–linked immunosorbent assay (ELISA), gas–chromatography mass–spectroscopy (GC–MS) and a recombinant yeast estrogen reporter assay. As measured by GC–MS, background estrone concentrations were approximately 100 ppb, while 17α–estradiol concentrations were one–third of the estrone concentration, and 17β–estradiol concentrations were below the detection limit (10 ppb). In contrast, background 17β–estradiol concentrations as measured by ELISA were 53 ppb. In press cake samples spiked with 17β–estradiol, ELISA and GC–MS 17β–estradiol concentrations from all experiments were well correlated (r² = 0.93), although the ELISA values were higher than the GC–MS values. The yeast estrogen assay was also highly correlated with GC–MS results (r² = 0.94). The rates of total estrogen removal in press cake samples spiked with 500 ppb 17β–estradiol and incubated over a range of 5–50°C were characterized by a 1st–order decay constant (k). The k values increased with temperature and ranged from 0.029 d⁻¹ to 0.12 d⁻¹. Rate constants observed in unspiked press cake samples agreed with the values derived from the spiked samples. Over a 7–d period, acidification of samples (pH < 2) and storage at 5°C reduced 17β–estradiol losses to 15% and total estrogen losses to 17%, whereas unacidified samples lost 90% of 17β–estradiol and 40% of total estrogen. The results of this study strongly suggest the need for acidification and cold storage of environmental samples being tested for estrogens. In this study, no single assay met all the desirable criteria of speed, sensitivity (<1 ppb), and detection of both 17β–estradiol and estrone. Therefore, the use of multiple assays for the detection of environmental estrogens is warranted.

Keywords. 17 beta–estradiol, Estrone, Hormones, Water quality, Assay methods, Degradation kinetics.

Due to their central role in the reproductive biology of a wide variety of organisms, environmental estrogens and estrogen–mimics have been implicated in human and wildlife health problems (Nakamura, 1984; McLachlan and Arnold, 1996; Panter et al., 1998). Recent work has shown that biologically significant quantities of estrogens are present in the effluent from some municipal wastewater treatment plants (Purdom et al., 1994; Folmar et al., 1996; Harries et al., 1997; Desbrow et al., 1998; Routledge et al., 1998, Baronti et al., 2000). Because estrogens, including the potent 17β–estradiol, are present in animal wastes (e.g., Monk et al., 1975; Knight, 1980), estrogen emissions from concentrated animal agricultural operations may be environmentally significant. Several investigators have examined losses of 17β–estradiol from land–applied poultry litter (Shore et al., 1995; Nichols et al., 1997, Nichols et al., 1998; Finlay–Moore et al., 2000), yet little is known about the fate of estrogens in dairy waste (Shore et al., 1993). In particular, published data on estrogen concentrations in dairy wastewater and reports specifying dairy waste treatment methods to reduce environmental estrogen loads are lacking.

An examination of the estrogen production by dairy cows confirms their potential importance as a source of environmental estrogens. Human estrogen production, as received by municipal wastewater treatment plants, can be estimated as 9–12 µg d⁻¹ per capita, from data in Ternes et al. (1999a). With a population of approximately 300 × 10⁶, the estrogen flux from the U.S. human population would be on the order of 3 kg d⁻¹. Based on available data from Monk et al. (1975) and Hoffmann et al. (1997), total estrogen excretion rates approach 1000 µg d⁻¹ per cow (Bos taurus). With over 9 × 10⁶ adult dairy cows in the U.S. (Tennessee Agricultural Statistics Service, 1999), the mass flux of dairy cow–excreted estrogen could easily exceed 9 kg d⁻¹. However, estrogen fluxes from U.S. dairies are not necessarily more environmentally significant than are fluxes from humans. This is because the risk associated with manorial estrogen depends not only on efflux rates and threshold concentra-
tions, but also on factors such as its fate in the waste handling system, its transport to receiving bodies of water, and the chemical transformations that it undergoes. For example, municipal wastewater treatment plants typically remove 50%–90% of total estrogen (Desbrow et al., 1998; Ternes et al., 1999a; Baronti et al., 2000; Layton et al., 2000). While investigators have started to address these issues for poultry–excreted estrogens (Shore et al., 1995; Nichols et al., 1997; Nichols et al., 1998; Finlay–Moore et al., 2000), the fate of estrogen from dairy wastes is poorly documented. The environmental significance of these fluxes needs to be determined, and, if needed, management practices established to keep these fluxes at ecologically acceptable levels.

Measuring estrogen fluxes at dairies is challenging for a variety of reasons, including the relatively low concentrations at which they occur, the potential for significant storage losses, and difficulties in extraction from the waste in question. In this study, three methods of assaying 17β–estradiol were compared: a commercially available enzyme linked immunosorbent assay (ELISA) method, gas–chromatography mass–spectroscopy (GC MS), and a recombinant yeast reporter assay. In addition, estrogen transformation and losses in manure solids were quantified, and an estrogen–conservative sample storage method was established.

**MATERIALS AND METHODS**

**SAMPLE COLLECTION**

Fresh (<1–d old), solid samples were collected from a Vincent screw press (Model KP–6 w/2.3–mm screen, Vincent Corp., Tampa, Fla.) located at The University of Tennessee Dairy Experiment Station in Lewisburg, Tennessee. The 180–cow herd is housed in free–stalls, and the manure handling and disposal system is typical of dairies in the southeastern U.S. Specifically, manure is scraped into an agitated pit, the resulting slurry is passed through a solids separator, the liquid separator effluent (press liquor) is stored in a holding pond prior to land application, and the solid portion of the separator effluent (press cake) is used as bedding for the animals.

Press cake samples were collected directly from the screw press prior to each experiment. Sample volumes of 2–5 L were collected in a 19–L bucket and homogenized with an auger to reduce spatial variability. Samples were analyzed for total solids (TS) and volatile solids (VS), as well as for total Kjeldahl nitrogen (TKN) and total phosphorus (TP) content, using Standard Methods (APHA, 1995). The press cake contained an average of 23% total solids and 91% volatile solids, and average nutrient concentrations of 5.0 g TKN/kg (±0.37) and 1.5 g TP/kg (±0.11), on a wet basis.

**THE EFFECT OF TEMPERATURE ON TRANSFORMATION AND REMOVAL OF ESTROGENS FROM PRESS CAKE**

Press cake samples were placed in sealed plastic bags for transport to the lab; they were not weighed or acidified in the field. In the laboratory, samples were refrigerated overnight at 5° C. The following day, 2.5–g sub–samples of press cake were placed into 40–mL glass sample vials. Sub–samples were spiked with 5 mL of water containing 250 ppb 17β–estradiol (17β–estradiol, 98% pure, Sigma, St. Louis, Mo.). Spiked samples were stored at 5°, 15°, 30°, and 50° C. They were analyzed in triplicate on days 0, 1, 2, 4, 7, 14, and 21 using ELISA and GC/MS. Samples extracted immediately after spiking were considered to be tested on day 0. Estrone and 17β–estradiol concentrations were summed to give estrogen concentration at each time. These data were fit by a decaying exponential function (y = ae–kt) and the 1st–order decay constants were calculated, using commercially available plotting and curve–fitting software (SigmaPlot 2000, SPSS Inc.).

**ETHER EXTRACTION OF PRESS CAKE SAMPLES**

Ten mL of pesticide grade ethyl ether (Fisher Scientific International, Pittsburgh, Pa.) was added to the 40–mL sample vials and put on a reciprocating shaker for 1 hour. The vials were centrifuged for 5 min at 500 rpm to remove the suspended solids from the organic layer. The organic layer was then sub–sampled as follows: 20 mL for ELISA, 500 mL for GC–MS analysis, and 20–100 mL for the yeast estrogen assay.

**ELISA 17β–ESTRADIOL ANALYSIS**

Ether extracts (20 mL) were evaporated to dryness. Sample vials were capped and frozen at −20° C. At the end of each experiment, all samples were assayed on a single, 96–well microplate. The dried, frozen extract in each vial was warmed to room temperature, resuspended in reagent grade denatured alcohol (Fisher) at a 6:1 ratio with assay buffer, and quantified using the 17β–estradiol antibody following the manufacturer’s procedures (Assay Design Inc., Ann Arbor, Mich.). The ELISA had a 17β–estradiol detection range of 0.0096–30 µg/L. According to the manufacturer, the cross–reactivity of the kit to estrone was 4.6%; the cross–reactivity to 17α–estradiol was determined to be 0.04%. The intra–assay coefficient of variation (cv) at 17β–estradiol concentrations between 0.048 and 1.2 ppb was 4%–10%, based on 8 replications.

**ESTROGEN ANALYSIS BY GC–MS**

Ether extracts in GC vials (500 µL samples) were evaporated under a gentle stream of nitrogen. Samples were capped and stored at −20° C. The dried extract was resuspended with 450 µL of DMF (dimethylformamide, Pierce, Rockford, Ill.), and then 50 µL of BSTFA [N, O–bis(Trimethylsilyl)trifluoroacetamide, Pierce] was added.
to derivatize the estrogen compounds. The derivatization mixture was allowed to stabilize overnight at 25°C before being analyzed by GC/MS. Derivatized samples were analyzed on an HP 6890 Series gas chromatograph with a DB–5MS capillary column (J&W Scientific; 30 m × 0.25 mm i.d.; 0.25μm film thickness) using an HP 5973 mass selective detector. Helium was the carrier gas; a flow rate of 1 mL/min was used. A splitless injection method was used into a 260°C C Injector port. The time/temperature program started at 240°C for 2 min, then increased at 10°C/min to 300°C, where it was held for 10 min. Samples were analyzed twice. The first analysis, in the scan mode, was for compound identification by fragmentation pattern and retention time as compared to known standards. The second analysis, in the SIM (selective ion monitoring) mode, was used to quantify 17β–estradiol and estrone. The primary ions selected for quantification were m/z 416 for 17β–estradiol and m/z 342 for estrone. Estriol (m/z 504) was never detected in any samples; however, 17α–estradiol was detected in several samples (see discussion section). Standard curves of at least five points, ranging from 1–5000 ppb, were used for calculation of estrogen concentrations. Estrogen standards were from Sigma (St. Louis, MO). To verify correct primary ions and retention time, press cake samples spiked with 5000 ppb 17β–estradiol were extracted and analyzed.

YEAST ESTROGENIC ASSAY

A recombinant S. cerevisiae strain, containing the human estrogen receptor integrated into the yeast genome and with the estrogen–responsive sequences carried on a lacZ reporter plasmid (Routledge and Sumpter, 1996; Schultz et al., 1998), was used as a bioassay for estrogenicity. In this bioassay, referred to as the yeast estrogen assay, estrogen interacts with the receptor to activate the transcription of the lacZ gene, resulting in the production of the enzyme β–galactosidase. β–galactosidase activity is measured colorimetrically at 540 nm using the chromogenic substrate, chlorophenol red–β–D–galactopyranoside. The absorbances in 96 well microtiter plates were measured using a Packard Spectra Count plate reader with I–Smart 1.0 software (Packard Instrument Co., Meridian, Conn.). Cell growth in the microtiter plates was measured by absorbances; absorbance below 0.5 was indicative of sample toxicity. The yeast estrogen assays were run as described previously using 2–fold serial dilutions of 17β–estradiol starting at 625 ng/L (well concentration) both as a positive control and to construct standard curves (Schultz et al., 1998; Layton et al., 2000). The EC50 (half–maximum activity) of 17β–estradiol, the most estrogenic compound, was 49 (±13) pg/well. Estrone was 49.5% (±6.5%) as active as 17β–estradiol, while 17α–estradiol was only 8.0% (±2.1%) as active as 17β–estradiol.

RESULTS

ESTROGENS IN UNSPIKED PRESS CAKE SAMPLES

Three trimethylsilyl–derivatized estrogenic compounds were routinely detected by GC–MS in extracts from press cake spiked with 17β–estradiol and incubated for 3–7 d: 17β–estradiol (416 m/z, retention time 6.4 min), estrone (342 m/z, retention time 6.25 min) and 17α–estradiol (416 m/z, retention time 6.1 min) (fig. 1). The retention times for the three estrogens were verified using standards and comparison to an internal MS library (NIST, 1998).

The concentrations of estrone and estradiol in unspiked acidified samples at time 0 represent the background estrogen concentrations present in the press cake samples. By GC–MS, the average concentration of estrone was 98 (±18) ppb, 17α–estradiol was 32 (0.0 ± 1.6) ppb and 17β–estradiol was below the detection limit (<10 ppb). Other estrogens including estriol, estradiol sulfate and estrone sulfate, were not detected by selective ion monitoring (SIM) or scan modes. By ELISA, the background concentration of 17β–estradiol in unspiked acidified samples held at 5°C was 53 (±12) ppb. The discrepancy between the ELISA result and that of the GC–MS is thought to be due to cross–reactivity of the ELISA with estrone.

THE EFFECT OF TEMPERATURE ON THE TRANSFORMATION AND REMOVAL OF ESTROGENS FROM PRESS CAKE

Estrogen concentrations in spiked press cake samples over a 14–d period are shown in figure 2. For 17β–estradiol, ELISA results followed the same trend as GC–MS results (correlation between the two methods is discussed in detail below). At all temperatures, 17β–estradiol was rapidly removed during the first 24 h; estrone accumulated during the same 24 h. At all temperatures, estrone was removed more gradually than 17β–estradiol, with the greatest losses (90%) occurring at 50°C. Since estrone dominated the total estrogen, total estrogen removal followed the pattern of estrone loss, with rates of total estrogen removal increasing with increasing incubation temperatures. The rates of total estrogen loss were well fit (r² from 0.71 to 0.97) by a decaying exponential function, and the 1st–order decay constants increase with increasing temperature (fig. 3). These results indicated that storage of press cake samples at 5°C reduced losses of total estrogens, but did not prevent transformation of 17β–estradiol to estrone.
Figure 2. 17β-estradiol, estrone and total estrogen concentrations in press cake samples spiked with 500 ppb 17β-estradiol and incubated at 5°C, 15°C, 30°C, and 50°C.

THE EFFECT OF ACIDIFICATION AND TEMPERATURE ON TRANSFORMATION AND REMOVAL OF ESTROGENS FROM PRESS CAKE

Acidification of the press cake samples to pH < 2.0 and storage at 5°C was tested as a method to preserve 17β-estradiol. The ELISA assay indicated that significant amounts of 17β-estradiol were lost rapidly from the unacidified samples at either 5°C or 30°C (fig. 4A). In contrast, minor amounts of 17β-estradiol were lost from acidified samples at 5°C over 7 d. The large 17β-estradiol losses in unacidified samples (ca. 50%) at day 0 are likely to have occurred during the 2-h holding period between spiking and sample processing (fig. 4A).

The results from GC–MS confirm the ELISA results in demonstrating that 17β-estradiol concentrations decreased rapidly in non-acidified samples. However, the GC–MS data indicated that 17β-estradiol losses were associated with five-fold increases in estrone concentrations over back...
Figure 4. Estrogen concentrations in acidified and non-acidified press cake spiked with 500 ppb 17β-estradiol and stored at 5°C and 30°C, as measured by (A) ELISA, (B) GC–MS, and (C) yeast estrogen assay.

The previously estimated 1st-order decay constants were derived from spiked samples. To determine their applicability to unspiked samples, total estrogen losses from day 0 to day 7 in unacidified, unspiked samples held at 5°C and 30°C were used to estimate a 1st-order decay constant at both temperatures. At 5°C, $k = 0.029 \text{ d}^{-1}$, while at 30°C, $k = 0.077 \text{ d}^{-1}$; the $k$–values derived from the unacidified spiked samples are within 7% and 25% of these results, respectively (fig. 3).

**COMPARISON OF ASSAY METHODS**

A regression analysis was performed on the ELISA and GC–MS 17β–estradiol data sets from all experiments (fig. 5A). The resultant high correlation coefficient, $r^2 = 0.93$, indicated that the two methods were well correlated. The resulting slope and intercept (1.04 and 81 ppb) indicates that ELISA results were higher than GC–MS results. The expected relative estrogenic activity was calculated from GC–MS data as 17β-estradiol concentration plus one-half the estrone concentration for the comparison of GC–MS and yeast estrogen assay. The EC$_{50}$ value of 17β–estradiol was approximately 50 pg per well, or 50 pg per 10 µl assay sample (5 ppb). Samples with low concentrations of estrogens could be concentrated 2–fold; additional concentration frequently resulted in toxicity to the yeast. Regression analysis indicated a good correlation between yeast estrogen assay and GC–MS results ($r^2 = 0.94$), but with overprediction at low concentrations, and underprediction at high concentrations, as reflected in the slope (0.64) and intercept (36 ppb) values of the linear regression equation (fig. 5B). This is likely due to the sigmoidal shape of the estrogen receptor binding curve (Routledge and Sumpter, 1996).
Based on the GC–MS results, estrone was more abundant and persistent in the press cake samples than 17β–estradiol. In addition, literature suggests that estrone, despite its lower potency, may also be more environmentally relevant than 17β–estradiol, for three reasons. First, the primary estrogens excreted by domesticated ungulates (e.g., *Bos taurus*) are estrone and 17α–estradiol, and not 17β–estradiol (Ivie et al., 1986; Adams et al., 1994; Sawada et al., 1995; Vos, 1996; Hoffmann et al., 1997). Second, forms of estrogen other than 17β–estradiol, such as estrone, are biologically active in vertebrates, due to direct receptor binding and enzymatic transformation of estrone to 17β–estradiol. The relative estrogenic activity of estrone compared to 17β–estradiol, ranges from 50% to 100%, depending on the bioassay (Bhavnani and Woolever, 1991; Arcand–Hoy et al., 1998). Previous work with the yeast reporter assay used in this study showed it to be 50% as sensitive to estrone as to 17β–estradiol, and 10% as sensitive to 17α–estradiol as to 17β–estradiol (unpublished data). Third, estrone is more environmentally persistent than 17β–estradiol. For example, in wastewater treatment plant biosolids, 17β–estradiol was rapidly converted to estrone, while estrone was removed more slowly (Ternes et al. 1999b). In addition, estrone is present at a higher concentration than 17β–estradiol in river water and wastewater treatment plant effluents (Ternes et al., 1999a; Baronti et al., 2000; Rodgers–Gray et al. 2000). The data obtained from press cake spiked with 17β–estradiol also suggest that 17β–estradiol was rapidly converted to estrone, whereas estrone was slowly removed from the press cake samples over a period of several weeks.

In these experiments, glucuronide– and sulfate–conjugated forms of excreted estrogens may have been undetected. However, several studies indicate that glucuronide–conjugated estrogens are rapidly cleaved after elimination (Ternes et al., 1999b; Vos, 1996). Sulfate–conjugated estrogens may be more resistant to cleavage (Vos, 1996; Zhang and Henion, 1999). To determine whether conjugated estrogens were present in high concentrations, one set of acidified press cake samples was treated with sulfatase and glucuronidase (Type H–2 crude solution from *Helix pomatica*, obtained from Sigma) before extraction. The recovery of estrogens after enzymatic extraction was not significantly increased by these enzymatic treatments compared to untreated samples (data not shown), perhaps because endogenous enzymatic activity and acid treatment had previously cleaved most conjugated estrogens in the samples. Also, as previously noted, rate constants based on spiking with unconjugated 17β–estradiol agreed well with rate constants observed in unspiked samples, suggesting that the pool of conjugated estrogens in these samples was not sufficient to significantly change the rate constants for total estrogen removal.

An ideal assay for the detection of estrogens in manure would be low cost, rapid (total run time < 24 h), selective (i.e., capable of differentiating 17β–estradiol from estrone), and sensitive down to <1 ppb. Although GC–MS was selective, it is relatively slow, making it less useful for routine analysis of a large number of press cake samples. In addition, the cost of analysis and maintenance is high, in part because the columns and detectors are fouled by other organic components co–extracted in the ether phase. Furthermore, the detection threshold for estrone and 17β–estradiol is in the low ppb range. The yeast estrogen assay has been widely applied to determining the relative estrogenic activity of a variety of

**DISCUSSION**

Immunoassays specific for 17β–estradiol were the assays of choice in recent work measuring estrogen contamination in animal waste run–off experiments, presumably due to their specificity, sensitivity, low–cost and speed (Nichols et al., 1997; Bushee et al., 1998; Finlay–Moore et al., 2000). These immunoassays were initially developed for quantification of 17β–estradiol in tissue culture media, human saliva, urine, and serum. The application of these assays to animal wastes may be subject to cross reactivity to unknown compounds that may be abundant in the samples, such as plant steroids or pigmented compounds. In addition, these assays may be subject to cross–reactivity between 17β–estradiol and estrone, variations in kit quality between manufacturers, and errors inherent in colorimetric methods. For these reasons, two other methods, GC–MS and the biological yeast estrogen assay, were used to measure estrogens in press cake samples and validate data obtained from the ELISA.
xenobiotic compounds (Routledge and Sumpter, 1996; Schultz et al., 1998; Vinggaard et al., 1999), but less frequently for the quantification of estrogen in environmental samples. The main advantages of the yeast estrogen assay are that it represents biological activity and is less expensive than either GC–MS or ELISA. The GC–MS and yeast assay were well correlated in the 20–800 ppb range studied. In these studies, the detection limit of the yeast assay could not be extended below 1 ppb as further concentration of the samples was toxic to the yeast. Although the ELISA method may overestimate 17β–estradiol in samples with high estrone background concentrations, its low cost, assay speed, and sub–ppb sensitivity outweigh this disadvantage. Differentiation between the two estrogenic compounds should be attainable by employing an estrone ELISA in conjunction with a 17β–estradiol ELISA.

Conversion of 17β–estradiol to estrone and the subsequent removal of estrone are likely to be biologically mediated, due to the ability of acidification and low temperature to inhibit those processes. Interestingly, in spiked, unacidified samples incubated at 15°C and 30°C, 17β–estradiol concentrations increased again after their initial decline (fig. 2). This suggests that other conversion processes, such as transformation of estrone to 17β–estradiol, may also take place. Bacteria, in particular Cornybac terium, are capable of 17β–hydroxysteroid oxidation (17β–estradiol to estrone) and 17–hydroxy steroid isomerisation (17β–estradiol to 17α–estradiol) (Nixon et al. 1986). Thus biological processes would convert 17β–estradiol to estrone followed by removal of estrone. In contrast, if increases in 17β–estradiol were due to acid–catalyzed desorption of 17β–estradiol from solids, net estrogen (sum of 17β–estradiol and estrone) should have increased during this time; it did not.

CONCLUSION

The relative concentrations of estrogens in these press cake samples were estrone >17α–estradiol >17β–estradiol. As such, use of the 17β–estradiol ELISA method must be done cautiously, and the use of an estrone ELISA method in conjunction is warranted. However, in spiked samples, the 17β–estradiol ELISA was well correlated with GC–MS results. The yeast estrogen assay also correlated well with GC–MS and can provide a measure of predicted estrogenic activity. 17β–estradiol was highly labile in press cake samples — acidification and storage at 5°C was required to prevent significant losses of 17β–estradiol from occurring. Results strongly suggested the rapid conversion of 17β–estradiol to estrone occurred in unacidified, spiked samples. In these same unacidified, spiked samples, total estrogen concentrations (17β–estradiol + estrone) decayed following 1st–order kinetics, with rate constants increasing with temperature.

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