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Quantitative analysis of conjugated and free estrogens in swine manure: Solutions to overcome analytical problems due to matrix effects

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ABSTRACT

Although animal manure is an important source for environmental estrogens, quantitative analysis of estrogens in manure is complicated due to matrix interference. In the present study, chromatographic methods have been developed for quantification of conjugated and free estrogens in manure samples collected from pig farms. The whole manure samples, immediately after collection, were stored at 4 °C, acidified (pH ≈ 2.0) and spiked with (i) ^{13}C-labeled internal standards to account for possible storage related degradation and (ii) deuterium labeled internal standards for calibration and quantitative analysis. The liquid samples were extracted with ethyl acetate for separating conjugated and free estrogens. The solid samples were eluted with water for desorbing conjugated hormones followed by methanol for desorbing free hormones. The water and extracts were further purified using hydrophilic–lipophilic balance and/or aminopropyl cartridges. The conjugated estrogens were analyzed using high-performance liquid chromatograph–mass spectrometer, while the free estrogens were analyzed using gas chromatograph–mass spectrometer. The extraction and calibration methods used in the present study yielded excellent sensitivity, reproducibility and >85% recovery of both free and conjugated estrogens that was independent of the manure matrix. In general, the total estrogen loads in liquid and solid samples were 5.1 mg/l and 4.93 mg/kg, respectively. This may represent the hormonal load of approximately 2.3 tons estrogen per day

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1. Introduction

Environmental estrogens, even at low (ng/L) concentrations, are potent endocrine disruptors associated with adverse effects on reproductive biology of wild/domestic animals and cancer in mammals [1–4]. One of the perceived sources of environmental estrogens is animal manure (a liquid–solid slurry consisting of animal urine, feces, washing solutions, soil, food, plant parts, dead animal parts and microorganisms) and waste water that are either stored at the production site or applied to the agricultural fields [4]. In manure, estrogens occur in two forms: the free estrogens that are lipophilic and exhibits high estrogenic activity, and the conjugated estrogens that are water soluble and lack estrogenic activity. However, conjugated estrogens may be deconjugated by manure microorganisms, resulting in release of free estrogens and ensuing increase in estrogenic activity [5]. This suggests that conjugated estrogens may pose serious ecological consequences not recognized in the past due to lack of analytical methods to accurately measure conjugated estrogen concentrations in manure.

Although recent studies have provided valuable information regarding occurrence and fate of estrogens in animal manure, average estrogen concentrations reported in different studies vary widely [2,5–13]. This may be due to the differences in the estrogens' physicochemical characteristics [13], analytical procedures used and heterogeneity of the manure samples as described below.

Abbreviations: ^{13}C-gE2, ^{13}C-labeled estradiol glucuronide; ^{13}C-sE2, ^{13}C-labeled estradiol sulfate; fαE2, alpha estradiol-free; dfE2, deuterated free *beta* estradiol; ^{13}C-fE2, ^{13}C-labeled free estradiol; ^{13}C-IS, ^{13}C-labeled internal standard; dgE2, deuterated estradiol glucuronide; dsE2, deuterated estradiol sulfate; dfE1, deuterated free estrone; dgE1, deuterated estrone glucuronide; dsE1, deuterated estrone sulfate; dfE3, deuterated free estriol; dgE3, deuterated estriol glucuronide; dsE3, deuterated estriol sulfate; dIS, deuterated internal standard; gE2, estradiol glucuronide; sE2, estradiol sulfate; gE1, estrone glucuronide; sE1, estrone sulfate; gE3, estriol glucuronide; sE3, estriol sulfate; fE2, free *beta*-estradiol; fE1, free estrone; fE3, free estriol; IS, internal standard; fT, free testosterone; fP, free progesterone; gT, testosterone glucuronide; sT, testosterone sulfate.

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Table 1
Internal standards used in the present study.

Chemical	Natural analytes		Deuterated analytes	
	Source	$[MH]^+$ *m/z*	Source	$[dMH]^+$ *m/z*
fE2	Sigma	273	Cambridge	277
gE2	Sigma	447	C/D/N isotopes	451
sE2	Sigma	351	C/D/N isotopes	352
fE1	Sigma	271	Cambridge	273
gE1	Sigma	444	In house	448
sE1	Sigma	349	Cambridge	353
fE3	Sigma	308	C/D/N isotopes	311
gE3	Sigma	447	In house	406
sE3	Sigma	366.5	In house	370.5
fT	Sigma	288	C/D/N isotopes	291
gT	Cerillant	465	Cerillant	468
sT	C/D/N isotopes	368	C/D/N isotopes	371
fP	Sigma	314	Cerillant	320

(i) Manure contains variable proportions (5–85%) of solids and exhibit considerable heterogeneity in organic carbon (OC) and humic substances that influence distribution of hormones between the solid and liquid fractions, suppress estrogen's binding to soil and interfere with the signal in mass spectrometer [14–18].
(ii) Earlier studies have shown that estrogens were effectively degraded and/or transformed in environmental samples [19–22,11]. Transformation of fE2 to fE1 (that may reduce estrogenic activity) and *vice versa* (that may increase estrogenic activity) is one of the serious complications in quantification of estrogens and estrogenic activity [19,23].
(iii) Acid preservation of manure by H_2SO_4 and/or the use of internal standards resulted in reliable and accurate recovery of estrogens from samples stored at 4 °C [12,24]. However, acidification has also been shown to enhance estrogen–humic acid interaction resulting in further variability in estrogen recovery [25,26].

Therefore, the overall objective of this investigation was to develop and validate analytical methodologies that circumvent the problems associated with quantitative analysis of free and conjugated estrogens in pig manure. We describe (i) an extraction procedure using unique internal standards for each hormone, (ii) validation of the methodologies for direct analysis of free and conjugated estrogens using high performance liquid chromatography–mass spectrometry (HPLC–MS) method for conjugated estrogens and gas chromatography–mass spectrometry (GC–MS) method for free estrogens and (iii) quantification of estrogen concentrations in the solid and liquid fractions of manure.

2. Materials and methods

2.1. Materials

Free and conjugated estrogens were obtained from Sigma Chemical Co. (St. Louis, MO). β-Glucuronidase/sulfatase from *Helix pomatia* (Type H-2) was obtained from Sigma Chemical Co. (St. Louis, MO). Dansyl chloride (reagent grade), *p*-toluenesulfonhydrazide (reagent grade), and acetone (HPLC grade) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Dichloromethane, methanol (HPLC grade), formic acid, glacial acetic acid (HPLC grade), sodium bicarbonate, L-ascorbic acid, sodium hydroxide and sodium acetate were purchased from Fisher Scientific (Fair Lawn, NJ). Osais hydrophilic–lipophilic balance (HLB) and aminopropyl cartridges were from Sigma Chemicals. The ISs used in this study are listed in Table 1. The ISs not commercially available were synthesized *in house*. The following IS mixtures were used: (1) ^{13}C-IS mixture containing ^{13}C-fE2, ^{13}C-gE2 and ^{13}C-sE2 (750 ng/mL) for correction of storage-related degradation and (2) dIS containing dfE1, dfE2, dfE3, dgE1, dgE2, dgE3, dsE1, dsE2, dsE3 (750 ng/mL) for calibration and recovery determination.

2.2. Synthesis of internal standards

2.2.1. Synthesis of deuterium labeled estrogen glucuronides by enzymatic glucuronidation

Phosphate buffer (0.1 M, pH 7.1) was mixed with 0.3 mg of human liver microsomes (Sigma Chemicals, St. Louis, MO) and 15 mg of alamethicin (18 mg/0.3 mg microsome) and placed on ice for 20 min. Then, $MgCl_2$ (1 mM final), saccharolactone (5 mM final), and 10 mM dfE1, dfE2 or dfE3 were added. The mixture was incubated at 37 °C for 3 min then the reaction was initiated by adding UDPGA (5 mM final). The mixture was incubated at 37 °C for 30 min. The reaction was stopped with 25% ice-cold formic acid (without α-naphthyl β-D-glucuronide addition). After 30 min, the samples were centrifuged and conjugated estrogens were separated using a HLB solid phase column (Oasis columns, Waters) as described previously [3]. Conjugated estrogens were eluted with 1% methanol solution prepared in pure water and 0.5 mL fractions were collected. Each fraction was analyzed using HPLC–MS/MS (described later) and the fractions containing single analyte peak were pooled, freeze dried and stored at −80 °C for future use. Purity of the internal standards was checker periodically to ensure their integrity. If stored internal standards exhibited de-deuteration, either they were purified or new batches were prepared and validated.

2.2.2. Synthesis of deuterium labeled estrogen sulfates by enzymatic sulfation

Labeled free estrogens (10 nM in ethanol) were mixed with 50 mM Tris–HCl buffer (pH 7.4) solution containing adenosine 3′-phosphate 5′-phosphosulfate (25 μM), DTT (8 mM) and BSA (0.0625% w/v) in a volume of 200 μL. The reaction was started by the addition of recombinant SULT1E1 and continued for 45 min. Reaction was stopped with chilled chloroform (3 mL) and deionized water (1 mL). The samples were vortexed for 2 min and centrifuged at 1500 rpm for 10 min. The upper aqueous phase containing conjugated estrogens was collected. Conjugates estrogens were purified as described above.

2.3. Manure collection and processing

Whole manure samples (n = 36) were collected from facilities housing finisher pigs. The samples were acidified to approximately pH 2 with 2 N H_2SO_4 and spiked with 0.1 mL of ^{13}C-IS to account for possible storage-related estrogen degradation. A 0.5 mL aliquot of each sample was centrifuged at 3000 rpm and percentage of the solid content was measured. The samples were then classified into three groups according to their solid contents: <15% (low-solid manures, n = 7), >15% to 45% (medium solid manures, n = 20) and >45% to 85% (high-solid manures, n = 9). The samples were centrifuged and the solid and liquid fractions were separated (this resulted in 6 sets of samples). One part of each sample was analyzed immediately to establish ^{13}C-fE2, ^{13}C-gE2 and ^{13}C-sE2 baseline. Then, samples were frozen at −80 °C for further analysis. The ^{13}C labeled estrogens was used to correct the storage-related changes in hormone contents in manure.

2.4. Sample extraction (Fig. 1)

2.4.1. Manure liquid

Manure liquid samples were thawed to 4 °C and spiked with the dIS mixture containing 75 ng/mL of each estrogen. The samples were mixed with ethyl acetate (1:10 v/v), rotoracked for 30 min

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