Factors affecting estrogen excretion in dairy heifers

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ABSTRACT

Two studies were conducted to assess factors affecting estrogen excretion in dairy heifers. The objective of the first study was to quantify estrogenic activity in feces and urine during the estrous cycle. Ten non-pregnant Holstein heifers were fed the same diet for 28 d. Plasma, feces, and urine samples were collected daily. Plasma 17-β estradiol (17-β E_2) was quantified with RIA and used to confirm day of estrous. Feces and urine samples from days -12, -6, -2, -1, 0, 1, 2, 6, 12 of the estrous cycle were analyzed with RIA and Yeast Estrogen Screen (YES) bioassay. Plasma 17-β E_2 concentrations peaked on day of estrus, with feces and urine estrogenic excretion peaking a day after. The objective of the second study was to quantify variation in estrogenic activity in feces and urine due to increased dietary phytoestrogen content. Ten Holstein heifers were randomly assigned to treatment sequence in a two-period crossover design. Dietary treatments consisted of grass or red clover hay, and necessary supplements. Feces and urine samples were collected and pooled for analysis. Estrogenic activities of pooled samples were quantified using the YES bioassay. Estrogenic excretion in feces and urine was higher for heifers fed red clover hay. Fecal and urine samples from five heifers were analyzed using LC/MS/MS to quantify excretion of phytoestrogenic compounds. Heifers fed red clover hay excreted more equol than heifers fed grass. Identifying sources of variation in estrogenic activity of manure will aid in the development of practices to reduce environmental estrogen accumulation.

Key words: dairy heifer, estrogen, excretion, phytoestrogen

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ABBREVIATION KEY

17-β E_2: 17-β estradiol

17-α E_2: 17-α estradiol

AAW: apparently absorbed water

BMPs: best management practices

CPRG: chlorophenol red-β-D-galactopyranoside

E_1: estrone

E_2: estradiol

E_3: estriol

E_2-EDC: estrogenic endocrine disrupting chemical

E_2-eq: 17-β estradiol equivalents

EDC: endocrine disrupting chemical

ER: estrogen receptor

FSH: follicle stimulating hormone

FW: fecal water

FWI: free water intake

GnRH: gonadotropin releasing hormone

hER: human estrogen receptor

iNDF: indigestible neutral detergent fiber

LH: lutenizing hormone

LSM: least squares means

MCF-7: human breast cancer

RIA: radioimmunoassay

SDG: secoisolariciresinol diglycoside

TWI: total water intake

UO: urine output

YES: yeast estrogen screen

Chapter 1: Introduction

The United States Environmental Protection Agency declared in 2003 that animal feeding operations are a major environmental concern (EPA, 2003). Many of these animal feeding operations are under strict guidelines, such as the Effluent and Limitations Guidelines and Standards under the Clean Water Act, limiting their ability to manage wastes (EPA, 2003). Better understanding of the effects of diet and physiological status on estrogenic excretion will aid in developing practices aimed at reducing environmental accumulation of contaminants.

Among other contaminants, manure from dairy operations contains various hormones (Lange et al., 2002; Hanselman et al., 2003; Lorenzen et al., 2004). Hormones are chemicals synthesized from glands in the endocrine system, excreted in urine and feces, and can have potent environmental effects (Crisp et al., 1998). When these hormones enter surface water they may functionally alter or disrupt the endocrine system of those organisms exposed to them (Nichols et al., 1997; Finlay-Moore et al., 2000; Jenkins et al., 2006,). Such hormones are known as endocrine disrupting chemicals (Crisp et al., 1998). Estradiol is one of the most potent naturally secreted estrogens, and is produced by the ovaries. It is also widely recognized as being responsible for growth and reproductive impairment in aquatic species when discharged into water (Harrison et al., 1995; Crisp et al., 1998).

Characterizing estrogen excretion patterns by bovines will allow assessment of the impact of dairy farms on estrogen accumulation in the environment. Evaluation of the effects of physiological states and diet formulations on estrogen excretion is

necessary for development of best management practices, to reduce estrogen loss from dairies.

Chapter 2: Review of Literature

ESTROGENS

Estrogens are natural hormones synthesized and secreted by the gonads of a variety of different animals (Behl, 2001). Estrogens are responsible for the normal growth and development of the female reproductive tract. Estrogen controls the estrous and menstrual cycle and inhibits the anterior pituitary's production of gonadotropin (Gower, 1979).

As part of their four-ring molecular structure, natural steroidal estrogens contain an aromatic A- and D-ring structure from which key structural differences arise. These structural differences allow for the addition of functional groups on the C-16 and C-17 positions. Estrone (**E_1**) has one hydroxyl group on C-3, estradiol (**E_2**) has an additional hydroxyl group on C-17, and estriol (**E_3**) has additional hydroxyl groups on C-16 and C-17 (Figure 2.1) (Hanselman et al., 2003). Potency varies with chemical structure causing 17-β estradiol (**17-β E_2**) to be the most potent natural estrogen; E_1 has a relative potency to 17-β E_2 of 0.38 and E_3 has a relative potency to 17-β E_2 of 2.4×10^{-3} (Rutishauser et al., 2004). Structural differences exist between 17-α estradiol (**17-α E_2**), where the C-17 hydroxyl group is in the *trans* configuration, and 17-β E_2, where the C-17 hydroxyl group is in the *cis* configuration (Figure 2.1). Natural estrogens are slightly soluble in water, moderately hydrophobic, and are weak acids (Hanselman et al., 2003).

Synthesis

Commonly the term "estrogen" is used to describe the natural endogenous hormone, E_2, which is secreted by selected endocrine glands and travels through the

blood in relatively low concentrations to specific target tissues (Behl, 2001). The major sources of estrogen in mammals are the granulosa cells of the ovarian follicles, and during pregnancy, the placenta (Lange et al. 2002). Additional sources of estrogen are adipose tissue, the adrenal cortex, the hypothalamus, the kidney, the liver, and muscle (Fotherby, 1984). Estrogen is synthesized from cholesterol in two separate pathways as shown in Figure 2.2 (Bidlingmaier and Knorr, 1978).

Falck (1959) was the first to observe that different ovarian cell types interact to produce E_2. Granulosa cells in cows are capable of producing E_2 only when an aromatizable substrate is present or when co-cultured with thecal tissue (Hansel and Convey, 1983). E_2 results from the multi-step conversion of cholesterol to androstenedione in the theca interna cell, while androstenedione converts to E_2 in the granulosa cell (Hansel and Convey, 1983).

Estrogen production and secretion is pulsatile due to pulsatile secretion of gonadotropin releasing hormone (**GnRH**), lutenizing hormone (**LH**), and follicle stimulating hormone (**FSH**). Secretion of estrogen occurs via the hypothamalus-pituitray-ovary-uterus axis. Hypothalamic release of GnRH causes the release of FSH and LH from the anterior pituitary. FSH and LH then stimulate the granulosa and theca interna cells in the follicle of an ovary to synthesize E_2 and E_1 for release (Behl, 2001).

Secretion

E_2 is secreted from the ovaries in response to gonadotropin release from the pituitary (Behl, 2001). E_2 must be transported from the ovaries to its target tissue through the circulatory system. E_2 travels in two forms in the circulatory system, free and bound, with only a small percentage being in the free form (Bidlingmaier and Knorr,

1979; Behl, 2001). The majority of E_2 in the circulating blood is bound to β-globulin, a plasma protein that binds both E_2 and androgens (Bidlingmaier and Knorr, 1979). E_2 binding to these carrier proteins prevents random diffusion into organs, tissues, or blood vessels which would elicit inappropriate responses, and also prevents rapid metabolism (Bidlingmaier and Knorr, 1979; Behl, 2001). Once E_2 reaches its target tissues, it produces various effects on developmental, growth, and reproductive bodily functions (reviewed elsewhere, e.g. Crisp et al., 1998).

Estrous Cycle

Development of estrous behavior in the bovine is a result of the action of E_2 (Britt et al., 1986). Blood E_2 concentrations increase during proestrus and estrus and then rise again during the early luteal phase (Henricks et al., 1971; Hansel and Echternkamp, 1972; Glencross et al., 1973; Chenault et al., 1975; Ireland et al., 1984). One or two large follicles persist on an ovary during the transition from proestrus to estrus and for several days after ovulation to mid-diestrus. During metestrus the follicle present during estrus ovulates, while the other follicles present during diestrus undergo atresia (Dufour et al., 1972; Matton et al., 1981). A follicle with high concentrations of E_2 in its follicular fluid develops and is “estrogen-active”. The “estrogen-active” follicle is responsible for most of the E_2 secreted during metestrus and diestrus (Ireland and Roche, 1982; Ireland et al., 1984).

Metabolism

Once E_2 is released from the ovaries and reaches and affects the appropriate target tissues, it re-enters circulation in the blood stream for catabolism and excretion. Estrogens pass through a series of metabolic pathways in the kidney, liver, and

gastrointestinal tract (Zhao et al., 2008). E_2 and E_1 are catabolized to form catechol estrogens. Catechol estrogens catabolize to methoxyderivatives of the catechols with the action of O-methyl-transferases. These methoxyderivatives enter into a final metabolism step in which conjugating enzymes, glucoronyltransferases and sulfotransferases, catalyze formation of glucorindes and sulfates, increasing the solubility of the estrogenic compounds in water. This allows for excretion of conjugated estrogens in urine (Behl, 2001; Shore and Shemesh, 2003).

The estrogens that are not excreted in urine pass to the liver, concentrating in bile (Pearlman et al., 1947). In the liver estrogens undergo hepatic conversions including metabolism and conjugation. While in the liver estrogens can be converted into six families of compounds: glucuronides, catechols, sulfates, fatty acid esters, hydroxylated metabolites, and E_1 (Zhu and Conney, 1998). Once metabolized and conjugated via enterohepatic circulation, estrogens are excreted into the bile or are reabsorbed by the gastrointestinal tract. Enterohepatic circulation of estrogens delays excretion from the body allowing for additional metabolism of the estrogen by micro-organisms in the intestine and the intestinal mucosa (Fotherby, 1984).

Excretion

Estrogen excretion rates and routes differ by species. It is estimated that the United States dairy cow population excretes 80 tons of E_2 per year (2060 µg/cow/d), while the pig population excretes 8 tons E_2 per year (290 µg/pig/d), and the human population excretes 3 tons E_2 per year (30 µg/human/d) (Johnson et al., 2006). Furthermore, it has been estimated that land application of livestock manure accounts for greater than 90% of the total estrogens in the environment (Khanal et al., 2006).

Studies utilizing radiotracers have shown that cattle excrete 58% of estrogens in their feces and 42% in their urine, whereas swine excrete 4% in their feces and 96% in their urine, and poultry excrete 31% in their feces and 69% in their urine. In cattle, 17-α E_2, 17-β E_2, and E_1 account for more than 90% of the estrogens excreted in free or conjugated forms (Hanselman et al., 2003).

Total E_2 excretion by cattle is significant, but there is little quantification data available (Zhao et al., 2008). In the past, studies aimed at quantification of E_2 excretion were primarily to establish tests used for fertility control and pregnancy diagnosis. Studies focused on hormonal changes occurring during estrus and pregnancy in older animals (Hanselman et al., 2003). These studies often used “ambiguous quantification methods” (Hanselman et al., 2003) relying on colorimetric techniques, radioimmunoassay, bioassays, and enzyme immunoassays making determining a reliable value for estrogen excretion for cattle difficult (Table 2.1) (Hanselman et al., 2003; Johnson et al., 2006).

Estrogen Excretion and Physiological States

Hoffmann et al. (1997) characterized estrogen production and metabolism during bovine pregnancy. They observed that during pregnancy hormonal changes were significant enough to affect estrogen excretion in dairy cows. These hormonal changes resulted in relatively high concentration of E_2 measured in feces that were unexpected. The low blood plasma E_2 concentrations measured suggest that blood E_2 is not only conjugated but oxidized and epimerized prior to excretion. Their research illustrates the need for a parallel between lactation and estrous cycles of the bovine, while expanding current knowledge regarding E_2 excretion (Hoffmann et al., 1997).

Gaverick et al. (1971) addressed estrogen production and excretion differences with age. They observed that heifers excreted more 17-α E_2 and less 17-β E_2 during the estrous cycle than cows. Average excretion of E_1 and total E_2 in urine and E_2 concentrations in plasma were similar for heifers and cows (Gaverick et al., 1971). Excretion of total estrogen during the initial estrus for heifers was higher (59 ± 15 ng/mg creatinine) when compared to cows (27 ± 5 ng/mg creatinine) but excretion during the second estrus were similar (29 ± 9 ng/mg creatinine for heifers and 34 ± 10 ng/mg creatinine for cows). During the estrous cycle, heifers excreted 33% of total estrogens as 17-α E_2 and 27% as 17-β E_2. Cows excreted 21% of total estrogens as 17-α E_2 and 42% as 17-β E_2 during the estrous cycle (Gaverick et al., 1971). The differences in 17-β E_2 excretion, the major estrogen produced by the ovaries, between heifers and cows led Gaverick et al. (1971) to conclude that heifers metabolize estrogen more completely than cows at most stages of the estrous cycle.

ENDOCRINE DISRUPTING CHEMICALS

An endocrine disrupting chemical (**EDC**) is any chemical that has the capability to interfere with the production release, transport, metabolism, or elimination of the natural hormones in the body responsible for the regulation of developmental processes (Crisp et al., 1998). EDCs are from a variety of different chemical classes, including pesticides, synthetic hormones, natural plant compounds, and various waste products from industry (Carr and Norris, 2006). EDCs can accumulate in the environment in numerous ways including the human use of pharmaceuticals, use of pesticides in farming practices, and land application of manure from animal operations (Crisp et al., 1998). Once EDCs enter and accumulate in the environment they have the potential to cause abnormalities

in various wildlife species (Colucci et al., 2001). Growing concern surrounds accumulation of these EDCs in the environment, making identifying the sources of these contaminants neccessary.

Exposures to EDCs early in development causes feedback mechanisms to be absent in the mature individual producing severe reproductive and developmental impairments that hinder the individual (Crisp et al., 1998). Effects of exposure have been seen in numerous aquatic wildlife populations with a variety of species being affected. For instance hydroxylated polychlorinated biphenyls and estradiol cause sex reversal in male turtle embryos. Also, DDT has resulted in feminization of gulls in ovo. A third example is developmental exposure to environmental estrogen or antiandrogens altering ratios of estrogen and testosterone in alligators (Sonnenschein and Soto, 1998; McLachlan, 2001). While negatively affecting the organization of reproductive, immune, and nervous function of individuals, the sublethal effects of endocrine disruption leads to long term detrimental consequences in populations (Crisp et al., 1998). Though exposure to EDCs can cause detrimental effects in a population, it is clear that exposure during development results in the greatest impact on an individual (Welshons et al., 2003).

Estrogenic EDCs

EDCs that have estrogenic activity are among the largest groups of known endocrine disruptors. Estrogenic EDCs (**E2-EDCs**) are chemicals that act as hormone mimics through the estrogen receptor (**ER**) mechanism. For E2-EDCs to have a direct estrogenic effect in a cell, the cell must possess an ER. ERs can be present in a number of cellular structures including the nucleus, cell membrane, or cytoplasm

(Welshons et al., 2003). E_2-EDCs need to have a binding affinity for the subtype of the ER, α or β, in a particular cell (Welshons et al., 2003).

EDCs and the dairy industry

EDCs and their possible impact on the environment and animal populations is an active area of research area. With the United States Environmental Protection Agency still developing methods for detection and identification of EDCs, the sheer abundance of EDCs in the environment is unknown (Degen and Bolt, 2000). In a review by Colborn et al. (1993) chemicals, reported to have an endocrine disrupting effect and that are widely distributed in the environment, were summarized. These chemical classes included pesticides, fungicides, insecticides, nematocides, hormones (synthetic and natural), and industrial chemicals (Colborn et al., 1993). With over 87,000 compounds needing to be screened for EDC properties, the focus of research is on establishing a data base on endocrine disruptors such as the Endocrine Disruptor Knowledge Base (EDKB) created by the Food and Drug Administration, which lists over 3,000 individual EDCs (Tong, 2009). Among other contaminants, manure from dairy operations contains various hormones (Lange et al., 2002; Hanselman et al., 2003; Lorenzen et al., 2004). Furthermore, it has been estimated that land application of livestock manure accounts for greater than 90% of the total estrogens in the environment (Khanal et al., 2006). The percentage of EDCs that are estrogens in unknown, and more work needs to be done in this research area.

PHYTOESTROGENS

Phytoestrogens are naturally occurring elements of plants that initiate E_2-like effects in animal tissue, although classically defined as compounds exerting estrogenic

effects on the central nervous system, inducing estrus, and stimulating growth of the female reproductive tract (Figure 2.3). The E_2-like effects of phytoestrogens are induced by binding to an ER and induction of specific estrogen-responsive gene products (Mazur, 2000). Phytoestrogens bind to an ER and act as E_2 mimics and antagonists because of the molecular similarity of the phenyl ring to E_2 in mammals. Phytoestrogens act differently depending on the tissue, ER present, concentration of circulating endogenous E_2, and their agonist or antagonist nature (Beck et al., 2005).

The variety of phytochemicals classified as E_2-EDCs continues to rapidly expand (Vajda and Norris, 2006). In 1954 Bradbury and White classified 53 plants as having E_2 activity affecting estrus in animals. In 1975 Farnsworth and coworkers expanded that list to include more than 300 plants. Phytoestrogens are a class of phytochemicals researched due to reported negative reproductive effects in ruminant females, attributed to the low concentrations of phytoestrogens eliciting subclinical effects and clinical outbreaks of estrogenism (Smith et al., 1979; Adams et al., 1988; Adams, 1995).

Phytoestrogen classes

Phytoestrogens can be divided into four main classes: isoflavones, coumestans, stilbenes, and lignans. All of these are diphenolic compounds with structures similar to those of natural and synthetic E_2 (Kurzer and Xu, 1997; Cornwell et al., 2004). These structural similarities allow phytoestrogens to mimic or antagonize E_2, while adding to their diversity. The vast diversity of phytoestrogens causes each compound, in its particular class, to affect the E_2-mediated response in a number of ways (Cornwell et al., 2004).

Isoflavonoids

Isoflavonoids are the most well known of the phytoestrogens. They are comprised of a very large and distinctive subclass of flavonoids and include daidzein and genistein (Mazur, 2000; Cos et al., 2003; Ososki and Kennelly, 2003). Structurally different from other classes of flavonoids in that one phenyl ring has shifted from the C-3 position to the C-2 position, isoflavonoids are primarily found in the *Fabaceae* family, but have been found in the *Iridaceae* and *Euphorbiaceae* families in soy and red clover extracts (Mazur, 2000; Ososki and Kennelly, 2003; Cornwell et al., 2004). Isoflavonoids exist as glycosides, which are hydrolyzed in the gut or through processing, or as aglycones, resulting from the hydrolization of glucosides (Figure 2.4) (Cornwell et al., 2004). The new compounds that are formed from the metabolism of various isoflavonoids may have very different biological effects (Ososki and Kennelly, 2003).

Coumestans

Coumestans' estrogenic activity was first discovered by Bickoff et al. (1957). It has since been isolated from various clover species and alfalfa, with the potent phytoestrogenic coumestans being coumestrol and 4'-methoxycoumestrol, found in legumes and are especially high in various clover species. Coumestrol content varies in plant material due to plant variety, stage of growth, harvesting practices, disease presence, location, and occurrence of insect or fungal attack (Ososki and Kennelly, 2003).

Lignans

Although there are more than 200 naturally occurring lignans, lignans have not been as thoroughly studied as isoflavones and coumestans. They are present in a variety of foodstuffs including cereals, fruits, and vegetables (Mazur, 2000; Ososki and Kennelly, 2003). They have a low molecular weight, are chemically unique, and stable due to the dibenzylbutane skeleton and positioning of the phenolic group in the *meta* position of the aromatic ring (Ososki and Kennelly, 2003). Lignans, a plant phenol group, result from the joining of two cinnamic acid residues or their biogenetic equivalents (Figure 2.5) (Mazur, 2000). Plant based lignans convert to mammalian lignans once they enter into the gastrointestinal tract and undergo various bacterial actions resulting in the formation of enterolactone and enterodiol (Figure 2.5) (Kurzer and Xu, 1997). Lignans have not been shown to induce estrus, as other phytoestrogens do, but have other E_2 like actions due to the formation of mammalian lignans resulting in their classification as phytoestrogens (Kruzer and Xu, 1997).

Stilbenes

Stilbenes are produced through the phenylpropanoid-acetate pathway. The main dietary source of phytoestrogenic stilbenes is resveratrol from red wine and peanuts. Resveratrol has two isomers, *cis* and *trans*, but only the *trans* form is estrogenically active (Cornwell et al., 2004).

Phytoestrogens in feedstuffs

The ability of plant compounds to cause estrus in animals was first documented during the mid-1920s. However, it was not until the 1940s that red clover, a plant rich in

phytoestrogens, was said to have effects on the fertility of grazing sheep (Bennetts et al., 1946; Adams, 1995). Research has continued in the area but with minimal investigation into the effect that phytoestrogens have on the total E_2 activity of animal excretions. Past research quantifies particular phytoestrogens with chromatography methods following the introduction of HPLC in the late 1970s and GC in the 1980s (Wang et al., 1990; Gültekin and Yildiz, 2006; Zhang et al., 2007).

High concentrations of phytoestrogens in the diet have effects in numerous populations. For instance, a high dietary intake of phytoestrogens may be associated with a reduced incidence of breast and prostate cancer in humans (Smith et al., 1979). Also, soy-derived phytoestrogens in standard laboratory rat feeds have shown detectable long term effects (Sharma et al., 1992). A third example is that sheep grazing on red clover show subclinical estrogenism and reproductive losses in Australia and New Zealand (Adams, 1995). However, there is minimal data on the effect of phytoestrogenic diets in bovines. Moreover, research utilizing bioassays to evaluate feedstuffs has fallen to the wayside, although more sensitive bioassays than the Allen-Doisy technique (discussed later) are now available, including the Yeast Estrogen Screen (**YES**) bioassay (discussed later).

Soybeans

Soybeans (*Glycine max*) belong to the Fabaceae family of plants and have long been used as a food source (Ososki and Kennelly, 2003). Soybeans' isoflavones content was first recognized in the 1940s and has been extensively researched as containing phytoestrogens with the first isoflavones isolated being genistin (Walter, 1941; Mazur, 2000; Ososki and Kennelly, 2003). Since then numerous studies have

focused on the isoflavone content of soybeans (Murphy et al., 1982; Kiessling, 1984; Coward et al., 1993; Kaufman et al., 1997; Mazur, 2000). There is large variability in concentration and composition among different soybeans and soybean products (Murphy et al., 1982; Franke et al., 1995). Of more importance to the dairy industry there have also been numerous studies on the effects of industrial processing, such as roasting time and temperature, and extraction methods, on the isoflavone content of soy products (Coward et al., 1983; Barnes et al., 1994).

Red Clover

Red clover (*Trifolium pretense*) is a perennial fabaceous (bean-like) plant native to the Mediterranean, commonly cultivated in the United States to feed livestock and as a green manure crop (Booth et al., 2006). The main estrogenic compounds in red clover are daidzein, genistein, formononetin, and biochanin A, with the latter two being glycosides (Pope et al., 1953; Schultz, 1965; Adams, 1995; Booth et al., 2006). The concentration of isoflavones in red clover appears to be under genetic control; however, environmental factors also affect the concentration of isoflavones (Adams, 1995).

The first reports of reproductive impairments in livestock were made in Australia and New Zealand in sheep grazing on red clover pasture (Bennetts et al., 1946; Smith et al., 1979; Adams et al., 1988; Adams, 1995). Researchers concluded that formononetin is indirectly responsible for the reproductive impairments in livestock, symptomatic of clover disease (Millington et al., 1964; Adams, 1998). Clover disease is defined by clinical manifestation of symptoms including impaired fertility, uterine prolapse, inappropriate mammary development, and inappropriate lactation in unmated ewes and castrated males (Adams et al., 1998). Additionally, red clover silage

containing isoflavones has been reported to cause infertility in cattle (Kallela et al., 1984).

Extensive studies on the effect of red clover have been done in sheep; however, there is little research available for its effect on dairy cattle. The metabolism path of isoflavones in cattle is similar to that of sheep (Braden et al., 1971). Kallela (1968) observed changes in the reproductive tract of ovariectomized heifers grazing on red clover similar to the changes seen in sheep populations grazing on red clover pastures. Yet, cattle appear to be less sensitive to the effects of red clover since the permanent infertility seen in sheep has not been observed in cattle (Lightfoot, 1974).

The differences in response to phytoestrogens between sheep and cattle may be due to a more efficient metabolism of formononetin and its metabolites in cattle when compared to sheep. In addition conjugation of the isoflavones and other estrogenic compounds to glucuronic acid by the liver as a detoxification step present in cattle may reduce the plant's effect. More research in this area is needed (Shutt et al., 1967; Braden et al., 1971; Cox and Braden, 1974).

METHODS FOR DETECTING ESTROGENIC ACTIVITY

Radioimmunoassay

Radioimmunoassays (**RIAs**) were first developed by Solomon Aaron Berson and Rosalyn Yallows in the 1950s to measure insulin concentrations. Since their development, RIAs have been used in a number of ways in endocrinology research due to their ability to precisely measure hormone concentrations (Kimball, 2005). RIAs are based on an antigen-antibody reaction in which a radiolabeled antigen competes with endogenous antigen from the sample to bind to a specific antibody. Binding is largely

based on the assumption that the radiolabeled antigen has the same binding affinity for the antibody as the endogenous antigen. The bound antigen is then separated from the free antigen and the radioactivity of the sample is measured (Kimball, 2005).

The benefits of RIAs are that they have a greater sensitivity than other assays. Quantification of the antigen is in the picogram range using these high affinity antibodies. RIAs do have limitations, mainly their high cost due to the radioactive material used, cross-reactivity, and the ability to only detect one compound (Kimball, 2005).

Bioassays

Bioassays are useful tools in determining the estrogenic activity of a variety of samples. The Allen-Doisy test is the first of the bioassays created to measure estrogenic activity. Ovariectomized animals (rats and mice) are injected with the substance to be measured. Administration of the substance can be directly into the vaginal area, increasing the sensitivity by eliminating circulation and metabolism of the substance, or injected elsewhere in the body. A vaginal smear technique is utilized to determine the extent of vaginal cornification, resulting in a relative potency measurement. The use of this assay has declined due to its inability to differentiate between estrogens and their antagonists, while also having an unreliable time course (Allen and Doisy, 1923; Jordan et al., 1985).

A second bioassay used is the human breast cancer (**MCF-7**) cell line. The MCF-7 cell line is estrogen sensitive and proliferates in the presence of estrogen. The increase in cell numbers is the biological equivalent of the increase in mitotic activity in endometrium of rodents (Soto et al., 1992). The limitations of this assay are that

multiple exposures to the estrogenic compound are necessary to elicit a response and that sample throughput is slow (Breinholt and Larsen, 1998).

A third assay is the YES bioassay. This assay was developed by Routledge and Sumpter (1996) and has been further modified for use in dairy waste (Zhao et al., 2009). A recombinant yeast strain (*Saccharomyces cerevisiae*) containing the human estrogen receptor (*hER*) gene and a chromogenic reporter system are utilized for this assay. The *hER* gene has been integrated into the chromosome of the strain in which the estrogen response elements are located on an extrachromosomal plasmid and regulate the expression of a *lacZ* reporter gene.

When the estrogen receptor molecule binds with estrogens and estrogen-like compounds, it co-locates with the estrogen response elements, resulting in β-galactosidase production. β-galactosidase migrates out of the cell into surrounding media and is quantified using a chromogenic substrate called chlorophenol red-β-D-galactopyranoside (**CPRG**). CPRG turns from yellow to red in response to the reaction and intensity of the red color quantitatively indicates the amount of estrogenic activity. Using the YES bioassay yields information on the total estrogenicity of the sample as estrogenic equivalents (**E_2-eq**) in relation to 17-β E_2 (Routledge and Sumpter, 1996). The YES bioassay is similar in sensitivity but lacks the specificity that RIAs have. This lack in specificity allows for detection of multiple estrogenic compounds, unlike RIAs, which is what is environmentally important.

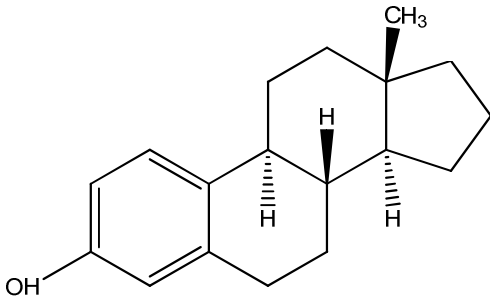
SUMMARY AND NEXT STEPS

Restrictions placed on waste management on animal operations and concern about environmental accumulation of EDCs resulted in a new body of research.

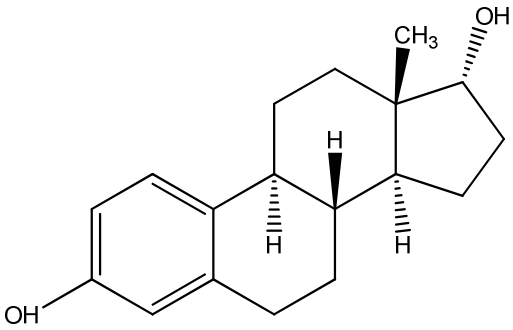
Estrogen, a hormone produced by cows and present in their manure, is one of the chemicals classified as an EDC. Additional research has led to classification of a variety of compounds, including phytoestrogens, which mimic estrogen and disrupt the endocrine system. Red clover is high in phytoestrogens and causes subclinical estrogenism and reproductive impairments. To assess estrogenic activity a variety of assays have been developed and improved over time. More work is needed to perfect and standardize the assays utilized to quantify estrogenic activity, as well as research on the impact of physiological state and feedstuffs on estrogen excretion. Therefore, the objectives of this research were:

1. To quantify excretion of estrogenically active compounds during the estrous cycle.
2. To determine the relationship between plasma 17-β E_2 and urine and fecal E_2-eq during the estrous cycle.
3. To quantify excretion of estrogenically active compounds due to feeding a high phytoestrogen diet.

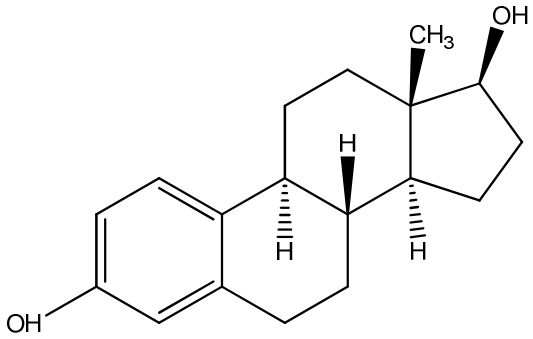
Figure 2.1 Structure of selected estrogens.



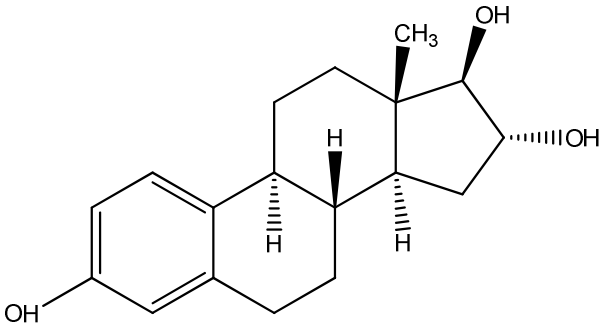
Estrone



17-α estradiol



17-β estradiol



Estriol

Figure 2.2 Biosynthesis of 17-β estradiol.

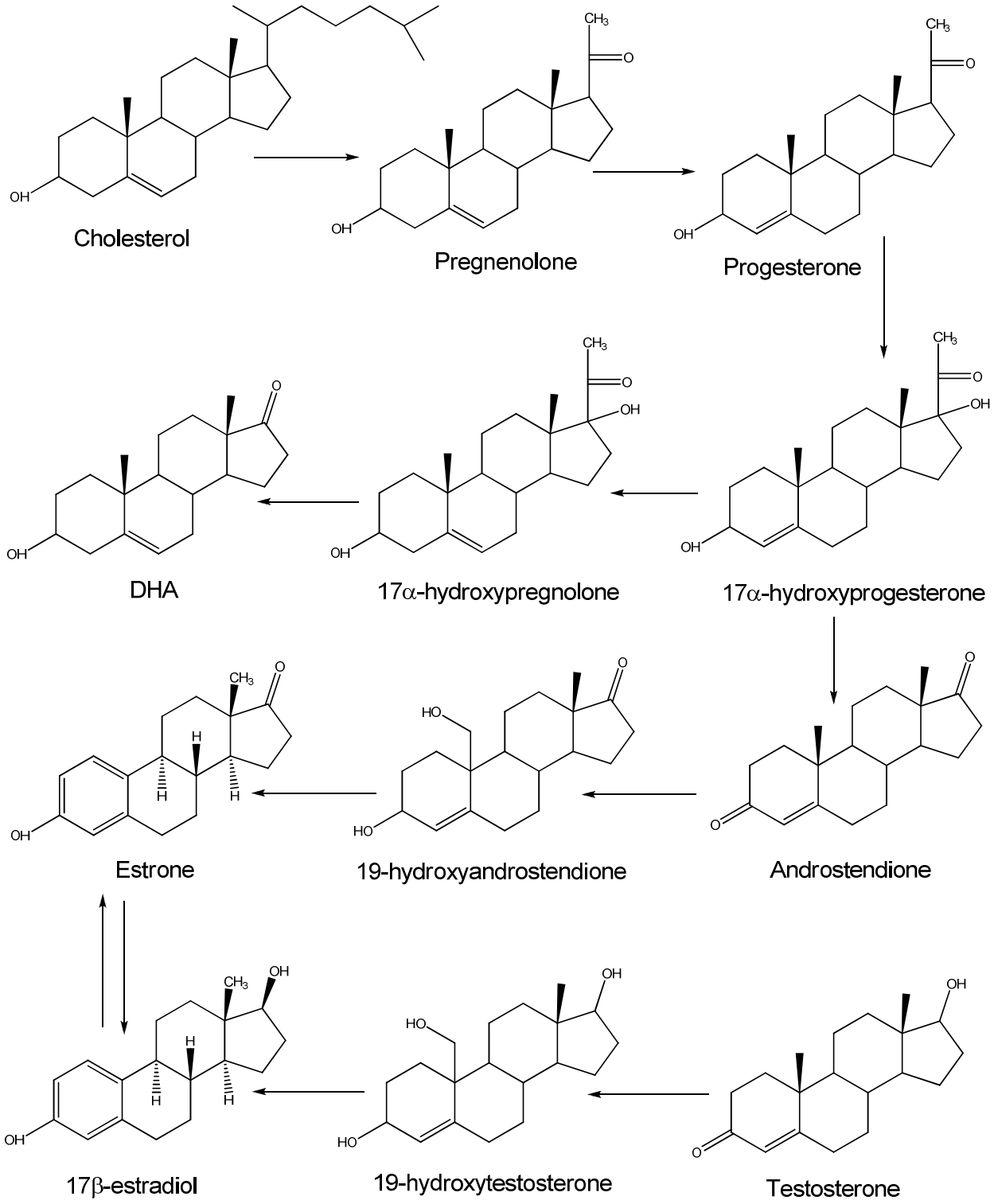
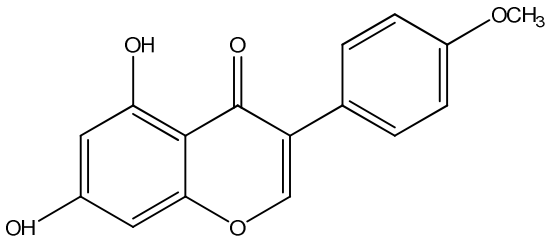


Table 2.1 Summary of research on estrogen excretion by cows.

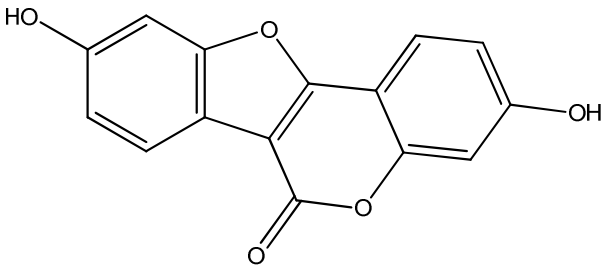
Study	Animal Status	Total Fecal Estrogens	Urinary Estrogens (µg/d/cow)
Erb et al., 1968a	Pregnant	NA[1]	E1 3600–28,800; E2β 1200– 3600
Erb et al. , 1968b	Pregnant and Ovariectomized	NA	11,300–31,464
Desaulniers et al., 1989	Cycling	34 ng/g	NA
Desaulniers et al., 1989	Pregnant	30 – 2000 ng/g	NA
Hoffman et al., 1997	Pregnant	10 – 1000 ng/g	NA
Möstl et al., 1997	Pregnant	10 – 180 ng/g	NA
Hanselman et al., 2003	Review	256 – 7300 µg/d/cow	320 – 104,320

[1]NA = Not Analyzed

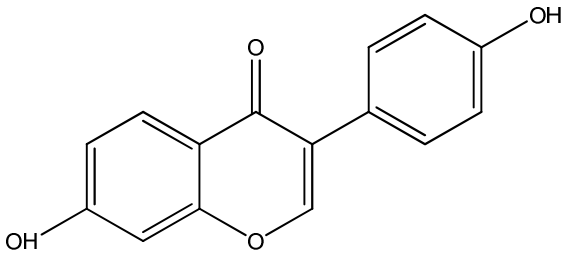
Figure 2.3 Structure of selected phytoestrogens.



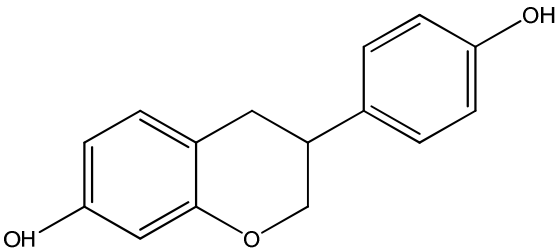
Biochanin A



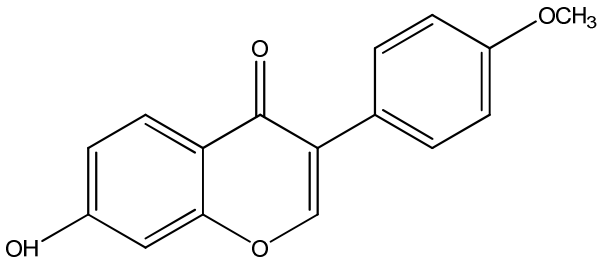
Coumestrol



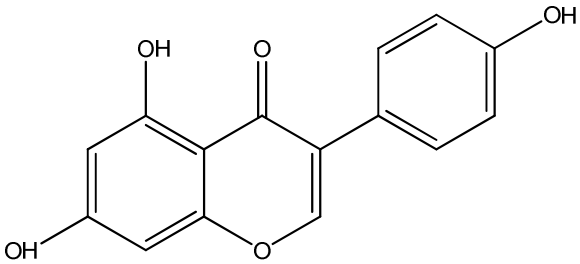
Daidzein



Equol



Formononetin



Genistein

Figure 2.4 Synthesis and degradation of the isoflavonoids daidzein and genistein.

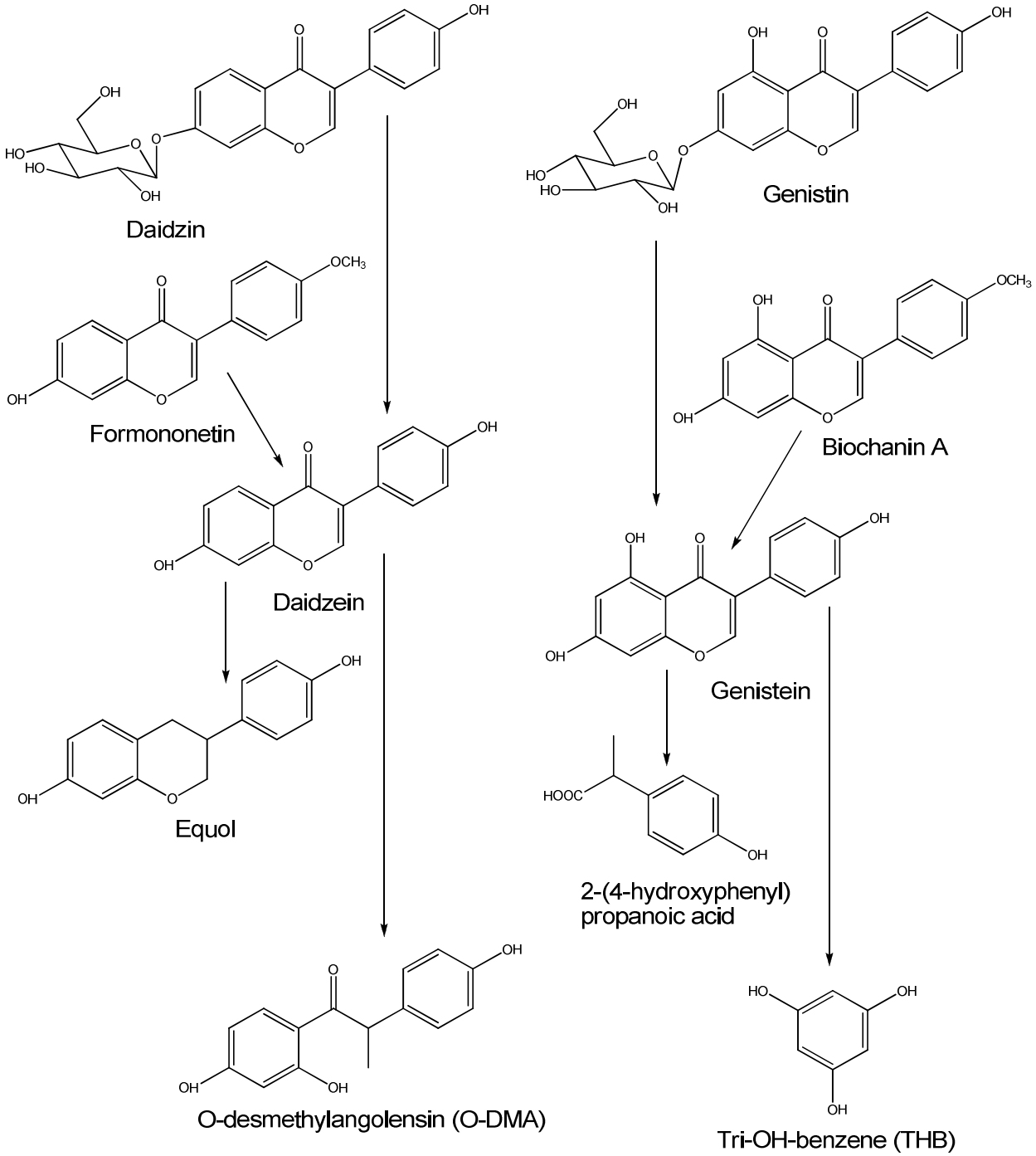
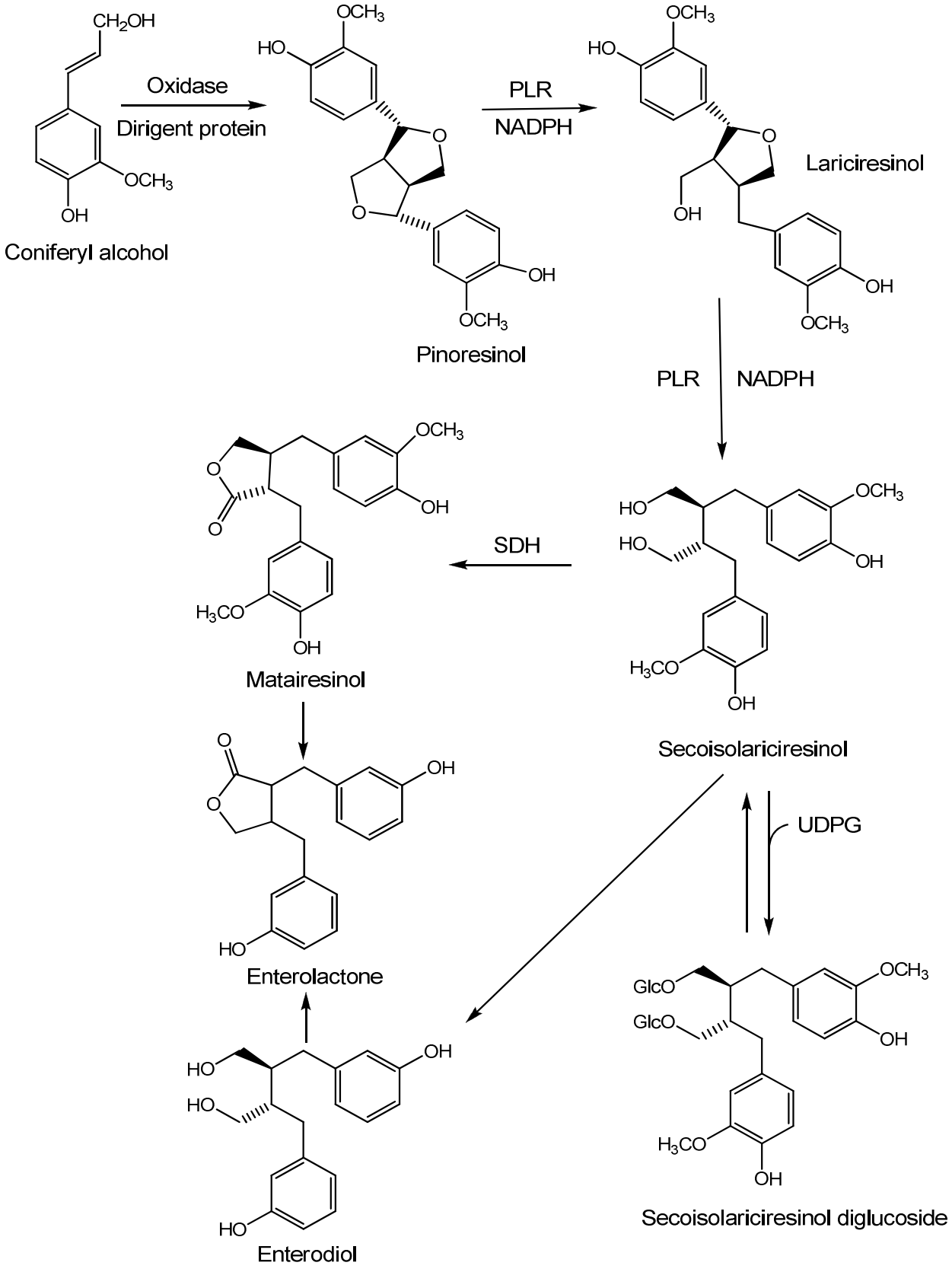


Figure 2.5 Biosynthesis and metabolic conversion of lignans.



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Chapter 3: Effect of the estrous cycle on fecal and urinary estrogen excretion

ABSTRACT

The United States Environmental Protection Agency has identified estrogens from animal feeding operations as a major environmental concern, but little data is available quantifying estrogen excretion by dairy cattle. The objectives of this study were to quantify estrogenic activity in feces and urine during the estrous cycle in dairy heifers, and evaluate relationships between excreted estrogens and plasma 17-β estradiol (17-β E_2). Ten non-pregnant Holstein heifers were fed a common diet for the 28 d study. Plasma was obtained via jugular venipuncture and grab samples of feces and urine were collected daily. Plasma 17-β E_2 was quantified with RIA and used to confirm day of estrous. Feces and urine samples from days -12, -6, -2, -1, 0, 1, 2, 6, 12 of the estrous cycle were analyzed with RIA and Yeast Estrogen Screen (YES) bioassay. The YES bioassay utilizes a recombinant yeast strain (*Saccharomyces cerevisiae*) containing the human estrogen receptor gene and a chromogenic reporter system to indicate total estrogenic activity. Plasma 17-β E_2 concentrations peaked on day of estrus (22.9 pg/mL), with feces and urine estrogenic activity peaking a day after plasma 17-β E_2 peaked. Excretion values mirrored sample concentrations, with fecal and urine estrogenic equivalent (E_2-eq) excretion peaking on d 1 of estrous (101 mg/d and 129 mg/d). Identifying sources of variation in estrogen excretion by livestock will aid in the development of practices to reduce environmental estrogen accumulation.

Key words: estrogen, estrous, feces, urine

INTRODUCTION

The United States Environmental Protection Agency declared in 2003 that animal feeding operations are a major environmental concern due to their concentration of environmental contaminants (EPA, 2003). Strict guidelines and standards make it necessary to develop practices that understand the effect of physiological status on estrogen excretion and to develop practices to reduce environmental accumulation of contaminants.

Hormones synthesized by glands in the endocrine system are excreted in urine and feces and may have potent environmental effects if runoff follows land application (Crisp et al., 1998, Lange et al., 2002; Hanselman et al., 2003; Lorenzen et al., 2004). When these hormones enter surface water they may functionally disrupt the endocrine system of organisms exposed to them, making them endocrine disrupting chemicals (**EDCs**) (Nichols et al., 1997; Crisp et al., 1998; Finlay-Moore et al., 2000; Jenkins et al., 2006). Estrogens are widely recognized as being responsible for growth and reproductive impairment in aquatic species when discharged into water (Harrison et al., 1995; Crisp et al., 1998,).

Limited data is available quantifying estrogenic excretions from dairy animals. Hoffmann et al. (1997) characterized estrogen production and metabolism during bovine pregnancy and observed hormonal changes over time significant enough to increase estrogen excretion 100 fold in feces and 550 fold in urine during pregnancy. More data is needed to characterize estrogen excretion, and total estrogenicity of manure, during other life stages of dairy animals. This data will assist in developing management practices to control estrogen loss from farms.

MATERIALS AND METHODS

Holstein heifers (n=10) averaging 13.3 ± 0.8 (SD) m of age and 290.8 ± 25.8 kg BW were fed in a Calan door system in a free stall barn (American Calan; Northwood, NH). During the 28 d study, all heifers received a common diet consisting of corn silage and a soybean hull and wheat midds based concentrate to meet the heifers' nutrient requirements (NRC, 2001; Table 3.1). Daily feed intakes were recorded. All procedures and protocols were conducted under the approval of the Virginia Tech Institutional Animal Care and Use Committee.

Sample collection

Samples of blood, feces, and urine were collected daily at 1100h. Blood samples were obtained via jugular venipuncture, alternating daily between the left and right jugular (Becton Dickinson Vacutainer; Franklin Lakes, NJ). Blood was stored on ice until centrifugation. Grab samples of feces were collected via manual removal from the ampula recti. Urine samples were obtained through induced micturition. All samples were placed on ice until sampling was completed.

Sample processing and analysis

Blood

Plasma was harvested via centrifugation (2000 x *g* for 30 min) and frozen at -80°C until analysis. At the conclusion of the study, plasma samples were thawed and extracted using ether (500 µL of plasma + 3.0 mL of ether) in 13x100 test tubes. Tubes were covered with plastic wrap, vortexed for 2 min, and frozen in a dry ice ethanol bath. The upper liquid ether phase was poured into clean tubes and evaporated using a

gentle stream of air (Oragnomation; Berlin, MA). The ether extraction was repeated, the sides of the tubes were rinsed using 0.5 mL of ether, and the extract and rinse were evaporated. Plasma extracts were re-constituted with 100 µL PBS-BSA and vortexed, covered, and stored overnight at 4°C.

Re-constituted plasma extracts were analyzed with ultrasensitive radioimmunoassay kit (DSL-4800) to determine concentration of 17-β E_2 (Diagnostic Systems Laboratories INC; Webster, TX). Resulting plasma 17-β E_2 concentrations were plotted by heifer to define individual estrous cycles. The day with the highest concentration of 17-β E_2 was declared d 0 of estrous. Fecal and urine samples from days -12, -6, -2, -1, 0, 1, 2, 6, and 12 of estrous were selected for further analysis (Figure 3.1). Three heifers were removed from the sample set because plasma RIA results indicated that they were pre-pubertal ([17-β E_2] < 4.3 pg/mL). Plasma 17-β E_2 pool size was calculated as the product of plasma 17-β E_2 concentration and estimated plasma volume (3.5% of BW; Dukes, 1955).

Feces

Feces samples were extracted on the day of collection using a base extraction technique described by Zhao et al. (2009). In brief, 10 g of wet feces were diluted by weight with 30 g of water. Aliquots (1.0 mL) of this mixture were placed into 4, 16x100 pyrolyzed glass test tubes and mixed with 1.5 mL of NaOH (1*N*) and 1.5 mL chloroform to solubilize the estrogens. Tubes were vortexed twice for 20 s and centrifuged (2500 x *g* for 20 min). The upper chloroform phases were pooled; 1.0 mL was aliquoted into 4, 16x100 test tubes and mixed with 180 µL of 90% acetic acid, to lower pH to 4.4, and 3.0 mL toluene. Tubes were vortexed twice for 20 s and centrifuged (2500 x *g* for 20 min).

Samples were stored overnight at -20℃ to enhance phase separation. The upper, toluene phase was decanted into clean tubes and stored at -20℃. The toluene extraction was repeated on the chloroform phase. Toluene phases were combined, evaporated to dryness using N_2 gas (Organomation; Berlin, MA) to concentrate the estrogens, and stored at -20℃. Samples from selected days (Figure 3.1) were analyzed with the RIA and YES bioassay described below.

Urine

Urine samples were extracted on the day of collection using the base extraction technique described by Zhao et al. (2009) and outlined above. Samples from selected days (Figure 3.1) were analyzed with the RIA and YES bioassay described below.

Yeast Estrogen Screen (YES) bioassay

Feces and urine samples were analyzed for total estrogenicity using the YES bioassay following the procedure developed by Routledge and Sumpter (1996) and modified for use in dairy waste (Zhao et al., 2009). In brief, a recombinant yeast strain (*Saccharomyces cerevisiae*) containing the human estrogen receptor (*h*ER) gene and a chromogenic reporter system was used to measure total estrogenic activity, expressed as 17-β E_2 equivalents (**E_2-eq**). In the recombinant strain, the estrogen response elements are located on an extrachromosomal plasmid and regulate the expression of a *lacZ* reporter gene, which produces β-galactosidase when transcription is activated. When the estrogen receptor element binds with estrogens and estrogen-like compounds in the sample of interest, the amount of estrogenic activity can be quantified by color change due to β-galactosidase (Routledge and Sumpter, 1996).

The assay was carried out in a laminar flow hood (Contamination Control INC; Lansdale, PA). Evaporated sample extracts were re-suspended with 1.0 mL of absolute EtOH. In sterile flat-bottomed 96-well microtiter plates (Becton Dickinson Labware; Franklin Lakes, NJ), 100 µL of the re-suspended sample was serially diluted and 10 µL of each serial dilution was aliquoted in triplicate. Plates were allowed to evaporate to dryness in air before 200 µL of assay medium containing yeast cells (grown to an absorbance of 1 at 620 nm) and chlorophenol red-β-D-galactopyranoside (**CPRG**) were added to each well (Holbrook et al., 2002). The plates were sealed and incubated at 32℃ for 24 h and then at room temperature for 12 h. Color density was quantified by measuring the absorbance at 575 nm and cellular density was quantified at an absorbance of 620 nm (µQuant BioTek Instruments, INC; Winooski, VT). Duplicates of each extracted sample were run on the same plate. Each plate also contained an E_2 standard curve (>98% purity, Sigma Chemical Company; St. Louis, MO) ranging from 976 to 125000 ng/mL.

Calculating daily excretion

Daily fecal output

Indigestible Neutral Detergent Fiber (**iNDF**) was used as a marker for fecal output. Feed and fecal samples were analyzed for iNDF (Cumberland Valley Analytical Services, Hagerstown, MD) using a 120 hr incubation period with a double inoculation of rumen fluid (Tilley and Terry, 1963). Fecal output was calculated with the equation:

$$\text{Fecal output (kg/d)} = \frac{\text{DMI (kg/d)x feed iNDF (\%DM)}}{\text{feces iNDF (\%DM)}}$$

Fecal density was measured using a water displacement technique (Lupton and Ferrell, 1986) and used to convert estrogen content (wt/vol) to excreted quantities. Daily fecal estrogenic excretion was then calculated as the product of fecal E_2-eq concentration and fecal output.

Daily urine output

Specific gravity was used to determine daily urine output as described by Holter and Urban (1992). Specific gravity of urine samples in duplicate acidified (1 mL of 12.1 *N* HCl per 80 mL of urine) samples was determined with a Midget Urinometer (Fischer Scientific) (Myers and Beede, 2009). Daily output of urine was calculated as:

Urine Output (**UO**):

UO (kg/d)= 212.1 + 0.8822 × DMI (kg/d) - 0.03452 × dietary %DM + 1.001 × dietary CP (%DM) - 216.4 × urine SG + 0.1414 × AAW
where:

Free Water Intake (**FWI**):

FWI (kg/d)= -10.34 + 0.2296 × dietary %DM+2.212 × DMI (kg/d) + 0.03944 × dietary CP (%DM)^2

Total Water Intake (**TWI**):

TWI (kg/d)= 35.19 + 0.9823 × FWI (kg/d) - 0.011 ×BW(kg) + 1.0817 × DMI(kg/d) + 1.184 × dietary CP(%DM) - 0.03881 × dietary CP(%DM)^2- 0.9963 × dietary %DM + 0.005488 × dietary %DM^2

Fecal Water (**FW**):

FW (kg/d)= 5.52 + 1.32 × DMI (kg/d) + 0.0384 x dietary %DM

Apparently Absorbed Water (**AAW**):

AAW (kg/d)= TWI – FW

(Holter and Urban, 1992)

Daily urinary estrogenic excretion was then calculated as the product of urinary E_2-eq concentration and urine output.

Statistical analysis

Plasma 17-β E_2 concentration, and concentrations and excretion of 17-β E_2 and E_2-eq in feces and urine were analyzed using the Mixed procedure of SAS (9.1, 2003) with the model:

$$Y_{ij} = \mu + H_i + D_j + e_{ij}$$

where:

μ = overall mean;

H_i = effect of heifer (i = 1 to 7);

D_j = effect of day of estrous cycle (d= -12, -6, -2, -1, 0, 1, 2, 6, 12); and

e_{ij} = error (heifer by day interaction)

Data are reported as least squares means (LSM) ± SE. Significance was declared at $P < 0.05$ and trends at $P < 0.10$.

RESULTS AND DISCUSSION

Estrogen and estrogenic activity

Plasma 17-β E_2 was low (< 2.5 pg/mL) throughout proestrus, metestrus, and diestrus and peaked at 22.9 pg/mL ± 2.4 (Effect of day $P < 0.0001$; Table 3.2, Figure 3.2); the day of peak plasma 17-β E_2 was deemed d 0 of estrous. Fecal 17-β E_2 and urine 17-β E_2 were not significantly different throughout the estrous cycle. Fecal and urine E_2-eq concentrations rose in the days leading up to estrus, peaked on d 1 of estrous (113 µg/mL ± 14; 108 µg/mL ± 13), and declined after d 1 of estrous (> 10

µg/mL). Patterns of daily excretion were similar to daily sample concentrations (Table 3.3, Figure 3.3), peaking at 101 mg/d ± 11 (feces) and 129 mg/d ± 16 (urine).

There is little published data on estrogen excretion by cattle during estrous. Using thin layer chromatography and gas liquid chromatography measurement techniques, Gaverick et al. (1971) observed that urinary estradiol (**E_2**) excretion was highest on day of estrus (42 ng/mg creatinine) and nearly as high on the day following estrus (37 ng/mg creatinine) and the 2 days preceding estrus (28 and 29 ng/mg creatinine). This pattern was not observed in the current study, with distinct increases in urine and fecal estrogenic activity on the day following estrus.

The observed pattern of plasma 17-β E_2 concentrations reflects expected ovarian release of E_2 in response to stimulation of the hypothalamus-pituitary-ovary-uterus axis to induce estrus, and the luteinizing hormone (**LH**) surge (Behl, 2001). During estrus, E_2 concentrations spike following ovulation of the Graafian follicle and then decline to low concentrations throughout the estrous cycle when gonadotropin concentrations are low (Senger, 1999). The pulse like release of LH and 17-β E_2 as well as secretion coinciding with the triphasic growth of the Graafian follicles may cause differences in estrogen excretion between animals (Cupps et al., 1959; Asdell, 1960; Bane and Rajakoski, 1961).

Secretion of estrogens from the ovaries varies during the estrous cycles. As part of their four-ring molecular structure, natural steroidal estrogens contain an aromatic A- and D-ring structure from which key structural differences arise. These structural differences allow for the addition of functional groups on the C-16 and C-17 positions. E_1 has one hydroxyl group on C-3, E_2 has an additional hydroxyl group on C-17, and

estriol has additional hydroxyl groups on C-16 and C-17 (Hanselman et al., 2003). Potency varies with chemical structure with 17-β E_2 the most potent natural estrogen. E_1 has a relative potency to 17-β E_2 of 0.38 and estriol has a relative potency to 17-β E_2 of $2.4x10^{-3}$ (Rutishauser et al., 2004). Interconversions occur between various estrogens, and their half-life varies, likely influencing the estrogenic activity of the manure following excretion.

Shaikh (1971) characterized secretion rates of estrone (**E_1**) and E_2 during the estrous cycle of rats. He observed that E_2 was secreted from the ovaries at a rate of 432 pg/h/ovary during proestrus and metestrus, increasing 11-fold during estrus, and increasing 4-fold during diestrus (Shaikh, 1971). Henricks et al. (1983) calculated the half-life of three types of estrogens in cattle: E_1, 17-α estradiol (**17-α E_2**), and 17-β E_2. E_1 has a half-life of 2.41 h, while 17-β E_2's half-life is 0.84 h, and 17-α E_2's half-life is 0.71 h (Henricks et al., 1983). Secreted estrogens are transformed during metabolism (details below) and these interconversions and differences in relative potencies of the estrogens likely influence the estrogenic activity of manure.

In pregnant cows, fecal estrogens have been reported to range from 256 to 7300 µg/cow/d (Hanselman et al., 2003). Using HPLC separation techniques and RIA quantification, Hoffman et al. (1997) reported that fecal concentrations of E_1, 17- α E_2, and 17- β E_2 excretion were low in the early days of pregnancy (~ 6 ng/g) and steadily increased with days of pregnancy, peaking at ~ 100 ng/g 5 d prior to parturition. Fecal 17-β E_2 concentration in feces (≤ 1.1 ng/g) were low during the early stages of pregnancy (-240 to -160 d before parturition). These values are similar to the fecal

concentrations reported by Möstl et al. (1984) (10 to 180 ng/g), and less than fecal concentrations reported by Desaulniers et al. (1989) (20 to 2600 ng/g).

Urinary estrogens range from 320 to 104,320 µg/cow/d (Hanselman et al., 2003). Erb et al. (1968) observed that total urinary estrogen excretion by pregnant cows increased throughout pregnancy from 123 to 3,402 µg/h/500 kg BW. Hoffman et al. (1997) observed low 17-β E_2 concentration in urine (< 0.002 ng/mosmol) during the early stages of pregnancy (-240 to -160 d before parturition), similar to the low concentrations observed in the current study.

Relative proportions of the different estrogenic compounds vary between urine and feces. Hoffman et al. (1997) reported that in urine, estrone was the primary excreted estrogen (89.9% of total estrogens), followed by 17-α E_2 (9.1%) and 17-β E_2 (1%). In feces 17-α E_2 was the primary excreted estrogen (56.7%), followed by 17- β E_2 (32%) and E_1 (11.3%). These differences account for the similarity in estrogenic activity of feces and urine samples despite the ~2 fold higher E_2 content of feces (Table 3.2).

Differences in quantification by RIA and YES

Observed 17-β E_2 concentrations in the urine and feces are lower than the E2-eq concentrations most likely due to hepatic metabolism. Estradiol is conjugated into six different families of compounds: glucuronides, catechols, sulfates, fatty acid esters, hydroxylated metabolites, and E_1 (Zhu and Conney, 1998). Hoffman et al. (1997) characterized urinary and fecal estrogen excretion during various stages of pregnancy. Utilizing HPLC separation techniques and RIA quantification, they observed that the estrone (89.9%) was the primary excreted estrogen, followed by 17-α E_2 (9.1%) and 17-β E_2 (1%). In feces 17-α E_2 (56.7%) was the primary excreted estrogen, followed by 17-

β E_2 (32%) and E_1 (11.3%) (Hoffman et al., 1997). 17-β E_2 concentration in feces (< 2.0 ng/g) and urine (< 0.002 ng/mosmol) were low during the early stages of pregnancy (-240 to -160 d before parturition), similar to the low concentrations observed in the current study. Estrone sulfate is the major urinary estrogen, while free estrogens are dominant in the feces (Hoffman et al., 1997).

The lag between peak plasma 17-β E_2 onset and peak urine and feces estrogenic activity may be due to typical metabolism of estrogen within the body. Estrogens are stored in the liver before passing to the kidney for excretion, or are conjugated and metabolized then pass into the gastrointestinal tract via bile. Kaltenbach et al. (1976) used radiolabeled E_2 (dpm/10g tissue converted to pg/g) to asses E_2 metabolism in heifers and observed greatest radioactivity in the liver and kidney (6,600 pg/g ± 1290, 4,740 pg/g ± 1390) and lower radioactivity in muscle (360 pg/g ± 50). Clearly the liver and kidney store and concentrate estrogens, releasing them in a pattern that lags behind rate of secretion. Bacterial conversion in the intestinal tract, entrance of estrogens into the bowel directly from circulation through the intestinal wall, and further conjugation and metabolism may also account for the lag pattern of excretion (Mellin and Erb, 1965).

Literature values are generally lower than those observed in the current study. This is largely due to the low sensitivity of earlier quantitative techniques, inadequate purification, and extraction methods (Mellin and Erb, 1965) and most importantly, differences in target compounds. The YES bioassay detects total estrogenic activity; while past studies quantified individual compounds with RIA or fluorometric assays. The appropriate quantification method measure depends on the goal of the study;

measurement of total estrogenic activity allows for assessment of relative environmental risk and may be more useful in development of best management practices (**BMPs**) to reduce estrogen losses from livestock farms.

CONCLUSIONS

Fecal and urine estrogenic activity peaked on d 1 of estrous, while there were no clear patterns for excretion of 17- β E_2. The disconnect between observed patterns of estrogenic activity in excreta during estrous and relatively consistent 17- β E_2 content in those samples is likely due to hepatic and gastrointestinal metabolism of estrogens to other compounds with estrogenic activity. Bioassay measurements of total estrogenic activity of manure reflect potential environmental risk more accurately than measurement of specific estrogenic compounds. Variation in manure estrogens with physiological status should be considered when instituting BMPs for control of estrogen losses from farms.

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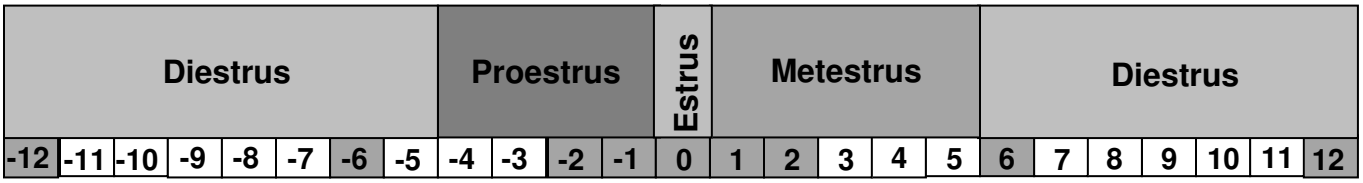
Table 3.1 Ingredient composition of diet.

Item	% of DM
Ingredients[1]	
Corn silage	35.5
Soybean hulls	19.4
Wheat midds	16.2
Cottonseed hulls	9.71
Corn gluten feed	4.85
Dried distillers grain	4.85
Corn, dried and ground	3.24
Molasses	1.94
Cottonseed meal	1.62
Soybean meal, high protein	1.62
Limestone	0.55
Salt	0.33
Vitamin and Mineral Mix[2]	0.16
Nutrient composition	
CP	14.5
NDF	46.6
ADF	28.3
Ca	0.6
P	0.5

[1] All ingredients except corn silage were combined to form a pre mix added to the corn silage daily.

[2] Contained: 43.6% Potassium/Magnesium Sulfate, 25% Selenium, 12.5% Vitamin E, 6.3% Bovatec, 160,000 mg/kg Zn, 150,000 mg/kg Mn, 4,000 mg/kg Cu, 3,500 mg/kg I, 1,600 mg/kg Co, 26,400 kIU/kg vitamin A, 8,800 kIU/kg vitamin D, and 4,400 kIU/kg vitamin E.

Figure 3.1 Sample selection for RIA and YES analysis. Shaded in grey are days of estrous cycle from which fecal and urine samples were analyzed.



Day of Estrous Cycle

Table 3.2 Effect of day of estrous on estrogen concentrations.

	Day of Estrous Cycle										
	-12	-6	-2	-1	0	1	2	6	12	SEM[1]	*P*-value
Plasma E_2 (pg/mL)	1.7	0.9	0.9	1.6	22.9	2.5	1.5	1.1	0.9	2.4	< 0.001
Feces E_2 (pg/mL)	10.8	11.4	11.8	14.1	13.6	14.5	23.5	19.4	21.5	1.6	0.87
Feces E_2-eq (µg/mL)	11	20	36	79	90	113	90	14	10	14	< 0.001
Urine E_2 (pg/mL)	4.9	4.9	3.4	8.1	4.9	5.5	3.4	4.8	3.2	0.5	0.49
Urine E_2-eq (µg/mL)	6	17	24	68	82	108	70	15	12	13	< 0.001

[1] SEM = standard error of the mean

Figure 3.2 Effect of day of estrous on estrogen concentrations in dairy heifers. ■ = Feces E_2-eq (µg/mL); ▲ = Urine E_2-eq (µg/mL); ♦ = Plasma E_2 (pg/mL).

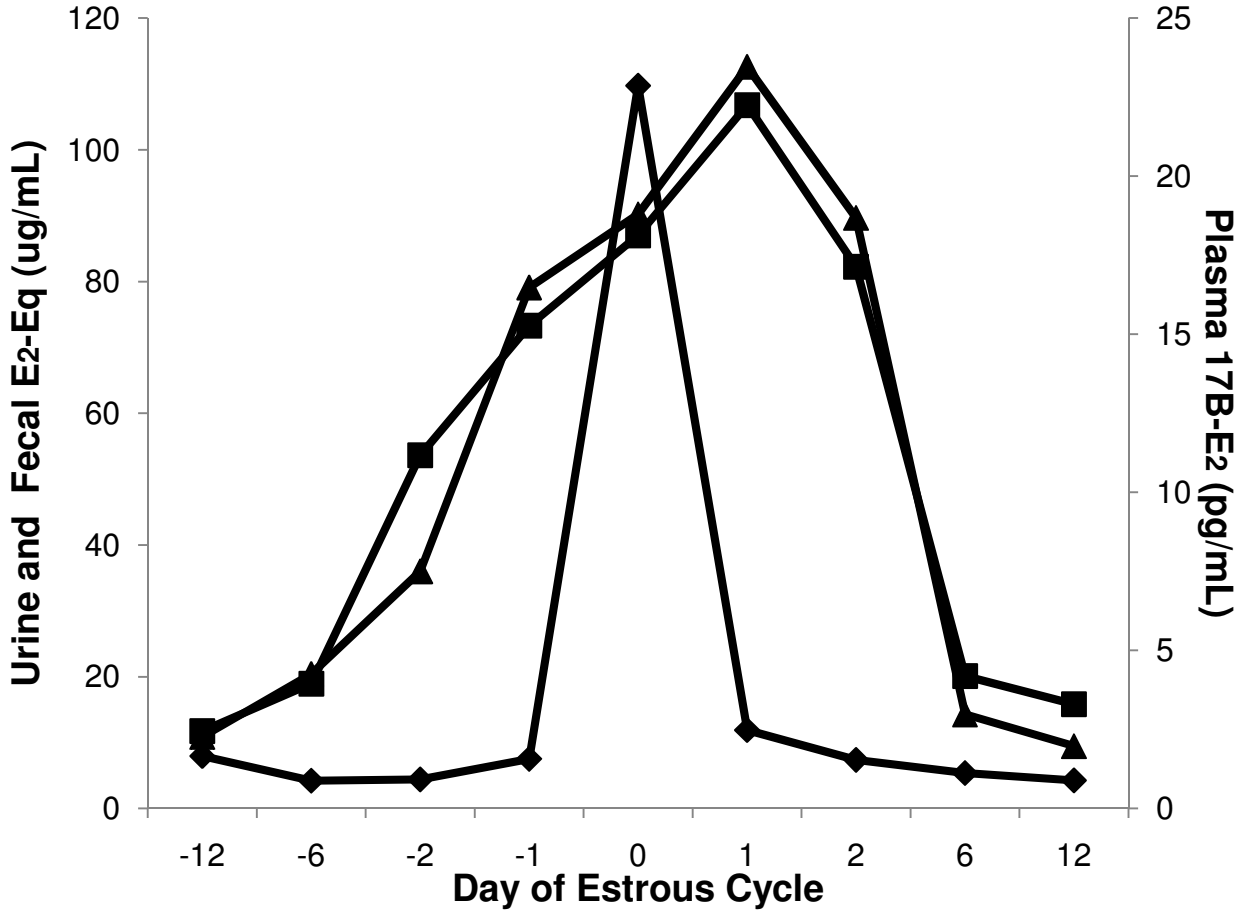


Table 3.3 Effect of day of estrous on estrogen excretion and 17-β pool size.

	Day of Estrous										
	-12	-6	-2	-1	0	1	2	6	12	SEM[1]	*P*-value
Plasma E_2 (ng)	19	11	11	18	262	27	18	13	9	15	< 0.001
Feces E_2 (ng/d)	10	12	15	17	18	17	29	20	24	2	0.88
Feces E_2-Eq (mg/d)	12	24	33	90	95	132	101	14	10	16	< 0.001
Urine E_2 (ng/d)	53	54	37	77	56	65	42	56	40	4	0.59
Urine E_2-Eq (mg/d)	7	21	30	82	101	129	88	18	15	15	< 0.001

[1]SEM = standard error of the mean

Figure 3.3 Effect of day of estrous on estrogen excretion and 17-β E2 pool size in dairy heifers. ■ = Feces E_2-eq (mg/d); ▲ = Urine E_2-eq (mg/d); ♦ = Plasma 17-β E_2 (ng).

Chapter 4: Effect of diet on fecal and urinary estrogen excretion

ABSTRACT

The United States Environmental Protection Agency has identified estrogens from animal feeding operations as a major environmental concern, but little data is available quantifying estrogen excretion by dairy cattle. The objectives of this study were to quantify variation in estrogenic activity in feces and urine due to increased dietary inclusion of phytoestrogens. Ten Holstein heifers were randomly assigned to treatment sequence in a two-period crossover design to evaluate the effect of diet on estrogen excretion. Dietary treatments consisted of grass hay or red clover hay, and necessary supplements. Total collection allowed for sampling of feed refusals, feces, and urine during the last 4 d of each period. Feces and urine samples were pooled by heifer and period and extracted using a base extraction technique, and estrogenic activity was quantified using the Yeast Estrogen Screen (YES) bioassay. Fecal and urine samples from five heifers were also analyzed using LC/MS/MS to quantify the excretion of specific phytoestrogenic compounds. Excretion of estrogenic equivalents in feces and urine was higher for heifers fed red clover hay (84.4 and 120.2 mg/d) when compared to those fed grass hay (57.4 and 35.6 mg/d). Liquid chromatography-double mass spectrometry analysis of feces indicated that heifers fed red clover hay excreted more equol, genistein, daidzein and formononetin (1634.0, 29.9, 96.3, 162.8 mg/d) than heifers fed grass hay (340.3, 3.0, 46.2, 18.3 mg/d). Diet had no effect on fecal biochanin A or coumestrol or any of the phytoestrogens detected in urine (2-carbethoxy-5, 7-dihydroxy-4'-methoxyisoflavone, daidzein and formononentin). Identifying sources of

variation in estrogenic activity of manure will aid in the development of practices to reduce environmental estrogen accumulation.

Key words: red clover, phytoestrogen, excretion, heifer

INTRODUCTION

The effect of dietary inclusion of phytoestrogens on estrogenic excretion needs to be researched to decrease estrogen losses for farms. High concentrations of phytoestrogens in the diet have negative reproductive effects in ruminant females, because of their ability to initiate estrogen-like effects in animal tissue (Smith et al., 1979; Adams et al., 1988, Sharma et al., 1992; Adams, 1995, Mazur, 2000). When these chemicals enter surface water they may functionally disrupt the endocrine system of organisms exposed to them, making them endocrine disrupting chemicals (**EDCs**) (Nichols et al., 1997; Crisp et al., 1998; Finlay-Moore et al., 2000; Jenkins et al., 2006). Phytoestrogens are widely recognized as being responsible for growth and reproductive impairment in aquatic species when discharged into water (Harrison et al., 1995; Crisp et al., 1998,).

Research on phytoestrogens is extensive but the effect of phytoestrogens on the total estrogenic activity of animal excretions has not been assessed. Shutt et al. (1970) observed that feeding red clover, a plant high in phytoestrogens, to sheep increased excretion of phytoestrogens in feces and urine. With Shutt et al. (1970) focused on metabolism of phytoestrogens in the rumen rather than excretion, minimal data is provided about estrogenic activity of the manure. Moreover, there is limited published work in cows and research utilizing bioassays to evaluate feedstuffs for estrogenic activity has fallen to the wayside, although more sensitive bioassays are now available. Therefore, the objectives of this study were to evaluate the effect of diet on the estrogenic activity of feces and urine, and to quantify the excretion of estrogenically active compounds.

MATERIALS AND METHODS

Holstein heifers (n=10) averaging 18.1 ± 1.7 (±SD) m of age, 103 ± 16.9 d pregnant, and 413.9 ± 44.5 kg BW were randomly assigned to a treatment sequence and balanced for days pregnant and age in a two-period crossover design. Dietary treatments consisted of grass hay or red clover hay, with a fixed amount of a byproduct-based concentrate to meet the heifers' nutrient requirements (NRC, 2001; Table 4.1). The experiment consisted of two periods, each lasting 14 d. The first 10 d served as an acclimation period to the diet followed by 4 d of total collection. During diet acclimation heifers were fed in a Calan door system in a free stall barn (American Calan; Northwood, NH) and feed offered was recorded daily. All procedures and protocols were conducted under the approval of the Virginia Tech Institutional Animal Care and Use Committee.

Total Collection

Total collection of feed refusals, feces, and urine was conducted during the last 4 d of each period, with four, 24 h total collections during each period. On d 9 of the diet acclimation period heifers were fitted with a urinary catheter (22 French, 75 cc; C.R. Bard, Inc., Covington, GA) and moved into metabolism stalls for a 24 h adaptation to both the metabolism stall and the catheter. The urinary catheter was connected to Tygon tubing (Saint-Gobain Performance Plastics, Mickelton, NJ) that drained into a sealed, clean plastic 12 L jug. Every 6 h, feces were removed from behind the heifer and placed into plastic containers. Feces and urine were weighed daily, mixed thoroughly, and sub-sampled. All excreta samples were stored at -20°C until analysis.

Feed offered and refused was recorded during the collection periods and feed was sampled on the final day of total collection.

Sample Processing and Analysis

Feed

Feed samples were collected on d 4 of total collection. Samples were dried at 60°C to a constant weights and then ground through a 1 mm screen in a Wiley mill (Arthur A. Thomas, Philadelphia, PA). These samples were then analyzed in duplicate for K, Cl, Na, P, Ca, and Mg (AOAC, 1984).

Feces

Daily fecal samples were pooled to one sample per collection period, weighted by daily excretion (wet basis) for each heifer, then extracted using a base extraction technique described by Zhao et al. (2009). In brief, 10 g of feces were diluted with 30 g of water. Aliquots (1.0 mL) of this mixture were placed into four, 16x100 pyrolyzed glass test tubes and mixed with 1.5 mL of NaOH (1*N*) and 1.5 mL chloroform to solubilize the estrogens. Tubes were vortexed twice for 20 s and centrifuged (2500 x *g* for 20 min). The chloroform phases were pooled; 1.0 mL was aliquoted into four test tubes and mixed with 180 µL of 90% acetic acid, to lower the pH to 4.4, and 3.0 mL toluene. Tubes were vortexed twice for 20 s and centrifuged (2500 x *g* for 20 min). Samples were stored overnight at -20°C to enhance phase separation. The toluene phase was decanted into clean tubes and stored at -20°C. The toluene extraction was repeated on the chloroform phase. Toluene phases were combined, evaporated to dryness using N_2 gas (Organomation; Berlin, MA) to concentrate the estrogens, and stored at -20°C.

Specific gravity of fecal samples was measured (Lupton and Ferrell, 1986) to convert estrogen content (wt/vol) to excreted quantities.

Urine

Daily urine samples were pooled to one sample per collection period, weighted by daily excretion (wet basis) for each heifer, then extracted using a base extraction technique described by Zhao et al. (2009) and outlined above. Specific gravity of urine samples was measured (Myers and Beede, 2009) to convert estrogen content (wt/vol) to excreted quantities.

Yeast Estrogen Screen (YES) Bioassay

Extracted feces and urine samples were analyzed for total estrogenicity using the YES bioassay following the procedure developed by Routledge and Sumpter (1996) and modified for use in dairy waste (Zhao et al., 2009). In brief, a recombinant yeast strain (*Saccharomyces cerevisiae*) containing the human estrogen receptor (*hER*) gene and a chromogenic reporter system was used to measure total estrogenic activity, expressed as 17-β E_2 equivalents (**E_2-eq**). In the recombinant strain, the estrogen response elements are located on an extrachromosomal plasmid and regulate the expression of a *lacZ* reporter gene, which produces β-galactosidase when transcription is activated. When the estrogen receptor element binds with estrogens and estrogen-like compounds in the sample of interest, amount of estrogenic activity can be quantified by color change by β-galactosidase (Routledge and Sumpter, 1996).

The assay was carried out in a laminar flow hood (Contamination Control INC; Lansdale, PA). Evaporated sample extracts were re-suspended with 1.0 mL of absolute EtOH. In sterile flat-bottomed 96-well microtiter plates (Becton Dickinson Labware;

Franklin Lakes, NJ) 100 µL of the re-suspended sample was serially diluted and 10 µL of each serial dilution was aliquoted in triplicate. Plates were allowed to evaporate to dryness in air before 200 µL of assay medium containing yeast cells (grown to an absorbance of 1.0 at 620 nm) and chlorophenol red-β-D-galactopyranoside (**CPRG**) were added to each well (Holbrook et al., 2002). The plates were sealed and incubated at 32°C for 24 hr and then at room temperature for 12 hr. Color density was quantified by measuring the absorbance at 575 nm and cellular density was quantified at an absorbance of 620 nm (µQuant BioTek Instruments, INC; Winooski, VT). Duplicates of each extracted sample were run on the same plate. Each plate also contained a 17-β E_2 standard curve (>98% purity, Sigma Chemical Company; St. Louis, MO) ranging from 976 to 125000 ng/mL. Daily fecal estrogenic excretion was calculated as the product of fecal E_2-eq concentration and fecal output. Daily urinary estrogenic excretion was calculated as the product of urinary E_2-eq concentration and urinary output.

LC/MS/MS analysis

Feces and urine samples from five heifers throughout the two periods, and samples of both hays from both periods were analyzed using LC/MS/MS. Feces and urine samples were pooled as before, freeze-dried (Freezone Plus 6, Labconco; Kansas City, MO), and ground using mortar and pestle. Hay samples were dried at 55°C for 48 h in a forced air oven and ground with a Wiley mill (1-mm screen; Arthur H. Thomas; Philidephia, Pa). Triplicate aliquots (0.5 g) of sample were mixed with hydramatrix (Varian, Inc; Palo Alto, CA) using mortar and pestle until the mixture was uniform. Samples were extracted in amber bottles using 50:50 (vol/vol) isopropyl alcohol/water (10 mL), vortexed, heated (45°C for 1 h), rotated (1 h), vortexed again,

and centrifuged (150 rpm for 10 minutes). The supernatant was decanted into a clean amber bottle and refrigerated overnight. The extraction was repeated, after which the supernatants were combined and evaporated to half the original volume with N_2 gas. 500 µL of the extract was transferred to vials (Agilent Technologies, Inc.; Santa Clara, CA) and stored until analysis. Samples were spiked according to a standard spiking procedure.

A liquid chromatograph (Agilent Technologies, Inc.; Santa Clara, CA) coupled to a Micromass Quatro Micro triple-quadrupole mass spectrometer (LC/MS/MS) (Waters Inc.; Milford, MA) was used for quantitative analysis of selected phytoestrogens: genistein, daidzein, equol, formononetin, coumestrol, biochanin A, and 2-carbethoxy-5,7-dihydroxy-4'-methoxyisoflavone. These phytoestrogens were selected for analysis based on reported presence in red clover hay and other feedstuffs. Two mobile phases were used (mobile phase A consisted of distilled/deionized water, while mobile phase B consisted of methanol) at a flow rate of 0.4 mL/min through the Waters Atlantis dC18 3µm, 30 x 150 mm column (Waters Inc.: Milford, MA). An isocratic pump was used after the liquid chromatography column with 10 mM ammonium hydroxide as a mobile phase flowing at a rate of 0.1 mL/min. MassLynx (Waters Inc.; Milford, MA) was used as the data acquisition interface.

Statistical Analysis

Estrogenic activity and excretion of E_2-eq in feces and urine

Concentrations and excretion of E_2-eq in feces and urine were analyzed using the Mixed procedure of SAS (9.1, 2003) with the model:

$$Y_{ij} = \mu + H_i + P_j + D_k + e_{ijk}$$

where:

μ = observed mean;

H_i = effect of heifer (i = 1 to 10);

P_j = effect of period (d= 1 to 2);

D_k = effect of diet (k= red clover, grass); and

e_{ij} = error (interaction of heifer and diet)

Data were reported as least squares means (LSM) ± SE. Significance was declared at $P < 0.05$ and trends at $P < 0.10$.

Concentrations of phytoestrogens in feces and urine

Concentrations of phytoestrogens in feces and urine were analyzed using the Mixed procedure of SAS (9.1, 2003) with the same model as above with one modification:

H_i = effect of heifer (i = 1, 2, 3, 4, 5)

Data were reported as LSM ± SE. Significance was declared at $P < 0.05$ and trends at $P < 0.10$.

RESULTS AND DISCUSSION

Effect of diet on estrogenic activity of feces and urine

DMI significantly increased with feeding of grass hay (P <0.01; Table 4.2). Feeding of grass hay significantly increased DM digestibility (P = 0.04; Table 4.2). Feeding red clover significantly increased urinary output when compared to grass hay (P < 0.01; Table 4.2). The effect of diet on urine output was likely due to higher protein content of red clover diets (13.7 vs 12.6%). The increase in urine output here (3.1 kg/d

with 0.9 unit increase in dietary protein) is somewhat greater than the increases of 1.0 to 1.9 L/d per percentage unit increase in dietary protein observed by Dinn et al. (1998) and Leonardi et al. (2003). Furthermore, increased concentrations of Na and K increase urine output because of the kidney's regulation of urinary excretion of these to maintain electrolyte homeostasis. (Berliner et al., 1950; Pickering, 1965). Concentrations of Na and K were higher in the red clover hay (0.08% of DM and 1.0% of DM) than in the grass hay (0.06% of DM and 0.81% of DM). Fecal output was not significantly affected by diet ($P < 0.7$; Table 4.2).

Feeding red clover hay significantly increased both urinary E_2-eq concentrations ($P = 0.01$; Table 4.3) and daily urinary excretion of estrogenic equivalents ($P = 0.01$; Table 4.3) when compared to grass hay. Similarly, feces from heifers fed red clover hay tended to have greater E_2-eq concentrations ($P = 0.06$; Table 4.3) and fecal excretion of estrogenic equivalents ($P = 0.08$; Table 4.3).

Limited data is available on the effect of dietary inclusion of red clover on estrogen excretion in feces and urine and no data is available on the effect of diet on E_2-eq excretion. Shutt et al. (1970) observed total phytoestrogen excretion in urine of 3820 mg/d for sheep fed red clover hay, while sheep fed subterranean clover (a less estrogenic legume) excreted 56 mg/d of total phytoestrogens in urine. Total phytoestrogen excretion in feces equaled 250 mg/d for sheep fed red clover hay, as compared to 11 mg/d for sheep fed subterranean clover (Shutt et al., 1970). Furthermore, substantial metabolism of specific phytoestrogens was observed. Formononetin was converted to equol in the rumen by the microbial population; this

conversion to equol resulted in high concentrations of equol in urine and the feces despite the lack of detectable equol in the diet (Shutt et al., 1970).

In the current study, intake of genistein, daidzein, and formononetin significantly increased with feeding of red clover hay while intake of biochanin A was higher in heifers fed grass hay (Table 4.5). Concentrations of daidzein and formononetin in feces tended to increase with feeding of red clover hay ($P = 0.07$; Table 4.4); daily fecal excretion of these two and of genistein significantly increased with feeding of red clover (Table 4.5). The concentration of equol and coumestrol in feces significantly increased with feeding of red clover although it was not detected in the diet (Table 4.4). Biochanin A and 2-carbethoxy-5,7-dihydroxy-4'-methoxyisoflavone were detected in feces but neither concentration nor excretion of these via feces were affected by diet (Table 4.4). Daidzein, equol, formononetin, and 2-carbethoxy-5,7-dihydroxy-4'-methoxyisoflavone were detected in urine but urinary concentrations and excretion of these were not affected by diet (Table 4.4, Table 4.5). Quantitative comparison of total excretion of specific compounds to intake of each compound suggests that equol and daidzein were produced at the expense of the other four measured compounds.

Thangavelu et al. (2008) observed increased excretion of three phytoestrogens (secoisolariciresinol diglycoside (**SDG**) and its metabolites, enterolactone and enterodiol) by cows with increased dietary inclusion of phytoestrogens. Quantification of the phytoestrogens was via GC-MS. Inclusion of flaxseed (10% of DM), a rich source of SDG, resulted in increases in fecal and urinary concentrations of SDG in cows. Fecal SDG concentrations averaged 34.7 µg/g for the flaxseed diet ($P < 0.01$), but did not increase SDG concentrations in urine. In humans and rats, dietary flaxseed increases

concentrations of all three compounds in feces and urine (Wang, 2002), but the complexity and difference in digestive physiology of ruminants make metabolic pathways of these phytoestrogens vastly different than those in monogastrics (Thangavelu et al., 2008).

Steinshamn et al. (2008) observed higher milk concentrations of biochanin A, equol, and formononetin in cows fed red clover silage than those fed white clover silage; the change in equol was most sizeable. Steinshamn et al. (2008) highlighted the lack of understanding of the mechanism of phytoestrogen recovery from feed.

There was a tendency for the effect of period to be significant in fecal E_2-eq concentrations ($P = 0.06$; Figure 4.1) and fecal excretion of estrogenic equivalents ($P = 0.08$; Table 4.3) occurred in the second period. Fecal equol concentrations tended to increase in the first period (Effect of period $P < 0.08$; Figure 4.1). The biological explanation for these is not apparent but undetected variation in the estrogenic activity. Period did not affect urinary concentrations of any of the compounds or estrogenic activity of urine.

Differences in quantification by YES bioassay and LC/MS/MS

Observed changes in content of specific phytoestrogens were reflected in changes in total estrogenic activity of feces and urine samples. The YES bioassay produced E_2-eq concentrations lower than the sum of the phytoestrogen concentrations quantified by LC/MS/MS (data not shown). This disparity may result from low binding affinities of the phytoestrogenic compounds in the samples to the mammalian expression vector used in the YES bioassay. Kuiper et al. (1998) determined relative binding affinities of several phytoestrogens relative to 17-β E_2. Compounds with the

highest relative binding affinity were coumestrol (73.5%) and genistein (26.2%). Daidzein, formononetin, and biochanin A had binding affinities less than 1%. These values are slightly higher than the relative binding affinities quantified by Shutt and Cox (1972) using cytosol of sheep uteri as the indicator: coumestrol (5%), genistein (0.9%), daidzein (0.1%), and equol (0.4%) The relatively low binding affinity of phytoestrogen to the estrogen receptor explains the difference in magnitude of estrogenic activity quantification by the YES bioassay.

Also estrogenic activity may be underestimated by the YES bioassay due to the presence of chemicals in the sample that are toxic or antagonistic to the yeast cells (Nakada et al., 2005). Nakada et al. (2004) observed that estrogenicity estimated by the YES bioassay was two- to ten-fold less than estrogenicity estimated by GC-MS analysis. They concluded fractionation occurring in the column chromatography separated any antagonistic or interfering chemicals from the estrogenic chemicals allowing for more accurate quantification (Nakada et al., 2005).

CONCLUSIONS

Feeding red clover to dairy heifers increases the estrogenic activity of feces and urine. The increase in dietary phytoestrogens resulted in increased excretion of five of seven measured phytoestrogens in the feces. The low recovery of phytoestrogens in the urine and feces while feeding red clover suggests that phytoestrogens are metabolized to other compounds in the digestive tract. The increased excretion of estrogenic equivalents in feces and urine of heifers fed red clover suggests that these metabolites retain estrogenic activity. Diet needs to be considered when instituting best management practices for control of estrogen losses from farms.

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Table 4.1 Ingredient composition of diet.

Item	Red clover diet[2]	Grass diet[2]
Ingredients[1]		
Red Clover Hay	84.7	--
Grass Hay	--	84.2
Wheat midds	8.21	8.21
Soybean hulls	3.48	3.48
Corn gluten feed	1.53	1.53
Dried distillers grain	0.77	0.77
Molasses	0.46	0.46
Limestone	0.33	0.33
Corn, dried and ground	0.19	0.19
Soybean meal, high protein	0.19	0.19
Vitamin and Mineral Mix[3]	0.151	0.151
Nutrient Composition		
CP	13.7	12.6
NDF	47.6	67.6
Ca	0.28	0.16
K	1.0	0.81
Mg	0.06	0.09
Na	0.08	0.06
P	0.33	0.27

[1] All ingredients except red clover and grass were provided as part of a pelleted supplement.

[2] % of DM

[3] Contained: 64.9% Salt, 13.26% Potassium/Magnesium sulfate, 7.28% Selenium, 5.30% Phosphate dicalcium, 160,000 mg/kg Zn, 150,000 mg/kg Mn, 4,000 mg/kg Cu, 3,500 mg/kg I, 1,600 mg/kg Co, 26,400 kIU/kg vitamin A, 8,800 kIU/kg vitamin D, and 4,400 kIU/kg vitamin E.

Table 4.2 Effect of diet on DMI, DM digestibility, and excreta output.

	Red Clover Diet	Grass Diet	SEM[1]	*P*-value
DMI (kg/d)	7.7	8.8	0.3	0.0089
DM digestibility (%)	58.3	62.6	1.0	0.04
Feces (kg/d)	22.1	21.5	0.8	0.67
Urine (kg/d)	11.9	8.8	0.6	0.0006

[1]SEM = Standard error of the mean

Table 4.3 Effect of diet on estrogenic activity and excretion of estrogenic equivalents in feces and urine.

	Red Clover Diet	Grass Diet	SEM[1]	*P*-value
Fecal E_2-eq (µg/mL)	3.92	2.64	0.4	0.06
Fecal E_2-eq (mg/d)	84.4	57.4	8.6	0.08
Urine E_2-eq (µg/mL)	9.89	3.78	1.6	0.01
Urine E_2-eq (mg/d)	120.2	35.6	20	0.01

[1]SEM = standard error of the mean

Table 4.4 Effect of diet on excretion of phytoestrogenic compounds in excreta as determined by LC/MS/MS.

	Red Clover Diet	Grass Diet	SEM	*P*-value
	----------*µg/g*----------			
Biochanin A				
Fecal excretion	0.27	0.20	0.06	0.55
Urinary excretion	ND[1]	ND	--	--
Coumestrol				
Fecal excretion	1.30	0.59	0.18	0.27
Urinary excretion	ND	ND	--	--
Daidzein				
Fecal excretion	4.56	2.14	0.58	0.07
Urinary excretion	0.06	0.06	0.01	0.75
Equol				
Fecal excretion	76.6	16.6	10.4	0.004
Urinary excretion	4.33	4.07	0.52	0.83
Formononetin				
Fecal excretion	8.28	1.0	1.7	0.07
Urinary excretion	0.12	0.09	0.01	0.21
Genistein				
Fecal excretion	1.41	0.33	0.21	--
Urinary excretion	ND	ND	--	--
2-carbethoxy-5,7-dihydroxy-4'-methoxyisoflavone				
Fecal excretion	0.07	0.01	0.03	0.52
Urinary excretion	0.61	0.29	0.19	0.52

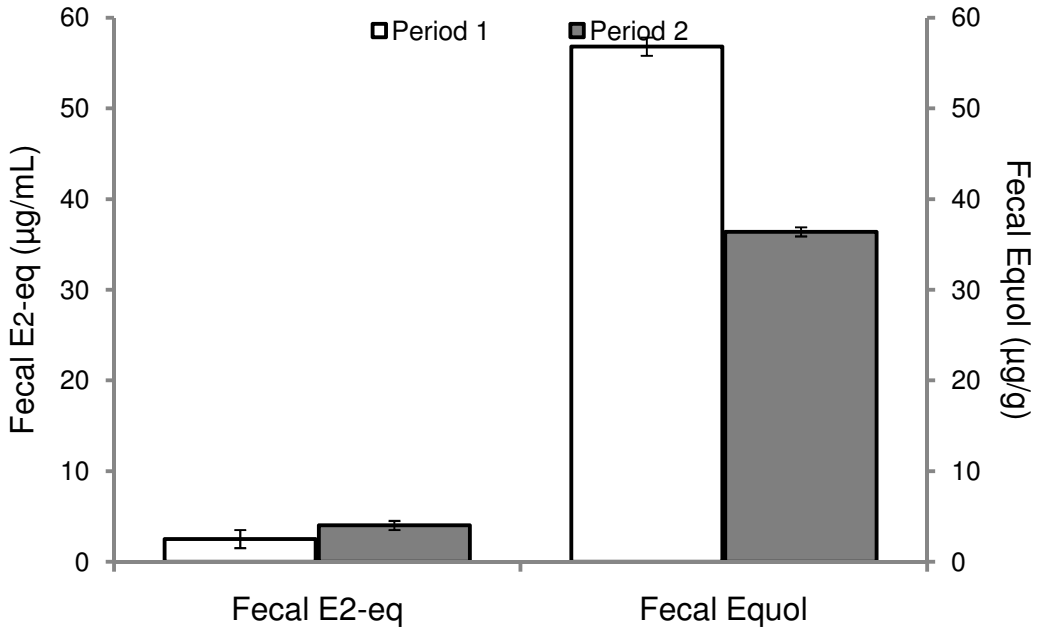
[1]ND = not detectable

Table 4.5 Effect of diet on phytoestrogenic compounds in feed and excreta as determined by LC/MS/MS.

	Red Clover Diet	Grass Diet	SEM	*P*-value
	----------*mg/d*----------			
Biochanin A				
Intake	31.8	77.8	12.9	0.004
Fecal excretion	5.7	3.7	1.0	0.25
Urinary excretion	ND[1]	ND	--	--
Coumestrol				
Intake	ND	ND	--	--
Fecal excretion	27.8	8.8	5.22	0.03
Urinary excretion	ND	ND	--	--
Daidzein				
Intake	158.1	34.2	21.9	0.002
Fecal excretion	96.3	46.2	12.8	0.04
Urinary excretion	1.3	1.3	0.3	0.85
Equol				
Intake	ND	ND	--	--
Fecal excretion	1634.0	340.3	233	0.006
Urinary excretion	49.7	34.9	7.3	0.27
Formononetin				
Intake	940.5	226.0	132	0.002
Fecal excretion	162.8	18.3	29.0	0.03
Urinary excretion	2.6	1.9	0.3	0.28
Genistein				
Intake	158.3	26.2	22.6	0.001
Fecal excretion	29.9	3.0	4.8	0.001
Urinary excretion	ND	ND	--	--
2-carbethoxy-5,7-dihydroxy-4'-methoxyisoflavone				
Intake	ND	ND	--	--
Fecal excretion	1.4	0.3	0.63	0.27
Urinary excretion	5.9	2.3	1.7	0.36

[1]ND = not detectable

Figure 4.1 Effect of period on fecal estrogenic activity. Data are reported as LSM ± SE. Period effect was significant for fecal E2-eq (µg/mL). Period effect had a tendency to be significant for fecal equol (µg/g) (*P* < 0.08).



Appendix A

PROTOCOL FOR ^{125}IODINE ESTRADIOL RADIOIMMUNOASSAY

I. Stock solutions

A. Buffers

1. 0.2M Monobasic Sodium Phosphate: Dissolve 2.76 g $NaH_2PO_4 \cdot H_2O$ in H_2O and dilute to 100 mL.
2. 0.2M Dibasic Sodium Phosphate: Dissolve 2.84 g Na_2HPO_4 in H_2O and dilute to 100 mL.
3. 0.01M Phosphate buffered saline with gelatin (PBS-gel)

1 liter	pH=7.2	**250 m**L
9 g	NaCl	2.25 g
1 g	Gelatin	250 mg
1 g	Sodium Azide	250 mg
14 mL	.2M NaH_2PO_4	3.5 mL
36 mL	.2M Na_2HPO_4	9 mL
9.16 mg	Phenol Red	2.29 mg

Add components to appropriate size beaker, dilute with slightly less than total volume of H_2O, adjust pH to 7.2, quantitatively transfer to appropriate size volumetric flask (i.e., rinse into flask with H_2O) and bring up to final

volume with H_2O.

4. Phosphate buffered saline with gelatin and bovine serum albumin (PBS-bsa). Weigh out 100 mg BSA (Sigma #A7030). Add a small amount of PBS-gel (just enough to wet the powder) and mash with a glass stirring rod to attain dissolution. Dilute to 100 mL with PBS-gel.

B. Standards (all standards should be stored at -20°C)

1. Stock A (100 µg/mL) - dissolve 10 mg E_2 in EtOH and dilute to 100 mL with EtOH.

2. Stock B (1 µg/mL) - dilute 1.0 ml Stock A to 100 mL with EtOH.

3. Stock C (100 ng/mL) - dilute 10.0 ml Stock B to 100 mL with EtOH.

4. Stock D (4000 pg/mL) - dilute 4.0 ml Stock C to 100 mL with EtOH.

C. Antiserum: Diagnostic Systems Laboratories Estradiol antibody (from Ultra-Sensitive Estradiol RIA kit, DSL-4800)

D. $^{125}I - E_2$ Diagnostic Systems Laboratories ^{125}I-Estradiol (from Ultra-Sensitive Estradiol RIA kit, DSL-4800)

E. Precipitating solution: Diagnostic Systems Laboratories Precipitating Reagent (from Ultra-Sensitive Estradiol RIA kit, DSL-4800)

F. Ether: Fisher Ethyl Ether Anhydrous #E138-4

III. Label tubes

A. Standards

1. One set of 12x75 glass tubes labeled: 1.5625, 3.125, 6.25, 12.5, 25, 50, 100, 200, 400

2. Two sets of 12x75 glass tubes labeled: T, 0, N, .15625, .3125, .625, 1.25, 2.5, 5, 10, 20, 40

Note: These values are pg/tube. Below 1.25 is often not measurable.

B. Samples

1. One set 13x100 glass tubes labeled 12 - 69

2. Two sets of 12x75 glass tubes labeled 12 - 69

IV. Working standards

Note: The following procedure will make enough standards for two assays. The volumes may be adjusted proportionately for more assays.

A. Pipette 100 µL Stock D (4000 pg/mL) into 12x75 tube labeled 400. Dry down under air.

B. Reconstitute with 1000 µL EtOH. Cover with parafilm and let sit for at least 1 h with periodic gentle vortexing.

C. Pipette 500 µL EtOH into 12x75 tubes labeled 1.5625, 3.125, 6.25, 12.5, 25, 50, 100 and 200.

D. Transfer 500 µL from the 400 tube to the 200 tube. Mix well. Repeat this process from the 200 tube to the 100 tube, the 100 tube to the 50 tube, etc. to finish the serial dilutions.

E. Pipette 100 µL aliquots from each tube into the two remaining sets of 12x 75 tubes (i. e. 100 µL of 400 into 40 etc.). Dry down under air. The T, N and 0 tubes get nothing at this point.

F. Reconstitute ALL tubes with 100 µL PBS-gel. Vortex well. Cover with plastic wrap and store at 4° C overnight.

V. Extraction

A. Set up Hamilton Digital Diluter for 3 mL ether and 500 µL sample

1. Ether - 5 mL syringe at 60%

2. Sample - 1 mL syringe at 50%

B. Dispense 3 mL ether only into first two 13x100 tubes (these are used to test for an ether blank effect)

C. Dispense ether and Low E_2 pool into next two tubes.

D. Dispense ether and High E_2 pool into next two tubes.

E. Dispense ether and samples into remaining tubes.

F. Cover with plastic wrap and vortex 2 minutes on the rack vortexer.

G. Freeze in dry ice alcohol bath (do **NOT** put in ultra-low freezer).

H. Pour off ether into one set of 12x75 tubes.

I. Dry down under gentle stream of air.

J. Re-extract with 3 mL ether; vortex 2 minutes; freeze; pour ether into same 12x75 tubes; dry under air.

K. Rinse sides of tubes with 0.5 mL ether and dry down.

L. Reconstitute all tubes with 100 µL PBS-bsa. Vortex well. Cover with plastic wrap and store at 4° C overnight.

VI. Assay

A. Reconstituted extracts from step K above were assayed directly

B. Combine standard and sample tubes into one rack.

C. Add 30 µL E_2 antibody to all tubes **except** T and N. Add 30µL PBS-gel to N tubes. Vortex. Incubate at room temperature for 1 h.

D. Add 50 µL ^{125}I-E_2 to **ALL** tubes. Vortex. Incubate at room temperature for 2 h.

E. Remove T tubes from rack and set aside. Add 1 mL precipitating solution to **ALL** remaining tubes. Vortex. Incubate in centrifuge at 4° C for 20 minutes.

F. Centrifuge at 2500g for 20 minutes at 4° C.

G. Decant supernatant to waste container and allow tubes to drain inverted on absorbent paper for 15 minutes. Gently blot the last drop onto dry absorbent paper.

H. Count tubes for 1 minute.

Appendix B

PROTOCOL FOR YEAST ESTROGEN SCREEN (YES) BIOASSAY

Preparation and storage of minimal media and medium components

Minimal medium and medium components prepared in glassware contaminated with an oestrogenic chemical will lead to elevated background expression. Glassware, spatulas, stirring bars, etc., must be scrupulously cleaned, and should not have had prior contact with steroids. Rinse glassware, spatulas, stirring bars twice with absolute ethanol, and leave to dry. Alternatively, wash twice with methanol, and once with ethanol.

Minimal Medium (pH 7.1)

Add 13.61 g KH_2PO_4, 1.98 g $(NH_4)_2SO_4$, 4.2 g KOH pellets, 0.2 g $MgSO_4$, 1 mL $Fe_2(SO_4)_3$ solution (40 mg/50 ml H_2O), 50 mg L-leucine, 50 mg L-histidine, 50 mg adenine, 20 mg L-arginine-HCl, 20 mg L-methionine, 30 mg L-tyrosine, 30 mg L-isoleucine, 30 mg L-lysine-HCl, 25 mg L-phenylalanine, 100 mg L-glutamic acid, 150 mg L-valine, and 375 mg L-serine to 1 L double-distilled water. Place on heated stirrer to dissolve. Sterilize at 121°C for 10 min, and store at room temperature.

D-(+)-Glucose

Prepare a 20% w/v solution. Sterilize in 50 mL aliquots at 121°C for 10 min. Store at room temperature.

L-Aspartic Acid

Make a stock solution of 4 mg/mL. Sterilize in 50 mL aliquots at 121°C for 10 min. Store at room temperature.

Vitamin Solution

Add 8 mg thiamine, 8 mg pyridoxine, 8 mg pantothenic acid, 40 mg inositol, and 20 mL biotin solution (2 mg/100 mL H_2O) to 180 mL double-distilled water. Sterilize by filtering through a 0.2-μm pore size disposable filter, in a laminar air flow cabinet. Filter into sterile glass bottles in 10 mL aliquots. Store at 4°C.

L- Threonine

Prepare a solution of 24 mg/mL. Sterilize in 10 mL aliquots at 121°C for 10 min. Store at 4°C.

Copper (II) Sulfate

Prepare a 20 mM solution. Sterilize by filtering through a 0.2-μm pore size filter, in a laminar flow cabinet. Filter into sterile glass bottles in 50 mL aliquots. Store at room temperature.

Chlorophenol red-β-D-galactopyranoside (CPRG)

Make a 10 mg/mL stock solution. Sterilize by filtering through a 0.2-μm pore size filter into sterile glass bottles, in a laminar flow cabinet. Store at 4°C.

Preparation and storage of yeast stock

Carry out all yeast work in a type II laminar flow cabinet.

Short term storage of yeast

Day 1

Prepare growth medium agar plate by adding 1 g of Difco agar to 90 mL minimal media. Autoclave the mixture and temper it. Add 10 mL glucose solution, 2.5 mL L-aspartic acid solution, 1.0 mL vitamin solution, 0.8 mL L-threonine solution, and 250 µL copper (II) sulfate solution. Pour agar mixture into 4 sterile plates. Allow plates to set

and place in 32℃ incubator for 24 hours.

Day 2

Obtain small amount of yeast from long term storage stock. Place one drop onto each of the four agar plates. Streak plates for isolation. Allow plates to dry slightly, invert, and place in 32℃ incubator. Once single colonies grow place plates in refrigerator for one month.

Long term storage of yeast

Day 1

Prepare growth medium by combinig 45 mL minimal media, 5 mL glucose solution, 1.25 mL L-aspartic acid solution, 0.5 mL vitamin solution, 0.4 mL L-threonine solution, and 125 µL copper (II) sulfate solution. Obtain one colony from short term storage agar plates and inoculate the growth media with it. Place media in 32℃ incubator until it reaches an absorbance of 1 (620nm).

Day 2

Transfer the culture to a sterile 50 mL conical tube. Centrifuge the conical tube for 10 minutes (4℃ at 2,000 x *g*). Decant the supernatant and resuspend the culture in 5 mL minimal media with 15% glycerol. Transfer 0.5 mL aliquots into cryovials, label, and freeze at -80℃.

Assay procedure

Carry out all yeast work in a type n laminar flow cabinet.

Day 1

Prepare growth medium by adding 5 mL glucose solution, 1.25 mL L-aspartic acid solution, 0.5 mL vitamin solution, 0.4 mL L-threonine solution, and 125 µL copper (II) sulfate solution to 45 mL minimal medium. Add 1 colony from short term storage agar plate. Incubate at 32℃ until it reaches an absorbance of 1 (620 nm).

Day 2

Serially dilute re-suspended samples and standard in 100 µL absolute ethanol in a 96-well microtitre plate. Transfer 3 - 10 µL aliquots of each concentration to a 96-well microtitre plate. Add 10 µL absolute ethanol (or appropriate solvent) to blank wells. Leave chemicals in the assay plate to evaporate to dryness.

Prepare growth medium by adding 5 mL glucose solution, 1.25 mL L-aspartic acid solution, 0.5 mL vitamin solution, 0.5 mL CPRG, 0.5 mL of yeast, 0.4 mL L-threonine solution, and 125 µL copper (II) sulfate solution to 45 mL minimal medium. Add 200 µl of the seeded assay medium to wells using a multi-channel pipette. Each assay should contain a plate with one row of blanks (solvent and assay medium only) and two rows of abiotics (assay medium without yeast added). Finally, each assay should have a 17β - estradiol standard curve. Seal the plates with autoclave tape and shake vigorously for 2 min. Incubate at 32℃ for 24 hours.

Day 3

Shake the plates vigorously for 2 min, to mix and disperse the growing cells. Place on bench top to develop further for 12 hours. Read the plates at an absorbance of

575 nm and 620 nm suing a plate reader.

Calculations

To correct for turbidity the following equation needs to be applied to the data:

Corrected value = chem. abs. (540 nm) - [chem. abs. (620 nm)-blank abs. (620 nm)]

Appendix C

PROTOCOL FOR LC/MS/MS ANLAYSIS OF PHYTOESTROGENS

Extraction Procedure for Phytoestrogen Samples

1. Weigh out 3 0.5 grams of sample into labeled aluminum pans.
    a. 1 = Unspiked
    b. 2 = Before Spike
    c. 3 = After Spike
2. To each sample add 0.5 hydramatrix and combine sample and hydramatrix using mortar and pestle until it is a uniform mixture.
3. Transfer samples to labeled 40mL amber bottles with septa.
4. Follow the spiking procedures to spike the unspiked, before spike, and after spikes.
5. Add 10mL of 50:50 (v/v) Isoprpyl Alcohol / Water to each sample. This is vial 1.
6. Vortex samples, and heat all samples at 45℃ for one hour.
7. While samples are heating obtain a second set of labeled 40mL amber bottles with septa and record weights as vial 2.
8. Vortex samples, rotate them for one hour, and vortex again.
9. Centrifuge samples at 1.5 rpm x 100 for 10 minutes.
10. Pour the top layer of the centrifuged samples into vial 2.
11. Add 10mL of 50:50 (v/v) Isoprpyl Alcohol / Water to each sample in vial 1.
12. Vortex samples, and heat all samples at 45℃ for one hour.
13. Vortex samples, rotate samples for one hour, and vortex samples.
14. Centrifuge samples at 1.5 rpm x 100 for 10 minutes.
15. Pour the top layer of the centrifuged samples into vial 2.

16. Follow the spiking procedures to spike the unspiked, before spike, and after spikes.
17. Weigh vial 2 and record.
18. Using nitrogen gas, blow each sample down to half the original weight of the extract.
19. Weigh each sample extract and determine which is the heaviest.
20. To each sample add enough water so that the weight of the extract equals that of the heaviest extract. Record the amount of water added and the final weight.
21. Transfer 500µL of each sample into labeled clear Agilent vials and place in freezer until ready to run.

Spiking Procedure for Phytoestrogen Samples

Before addition of the 10mL 50:50 (v/v) Isoprpyl Alcohol / Water:

To **ALL** Samples:

Add 100µL of the 1ng/µL LCHM surrogate standard

Add 200µL of the 1ng/µL LCPE d4-Genistein standard.

To the **BEFORE SPIKE** Samples:

Add 200µL of the 1ng/µL LCHM Analyte Standard Mix

Add 200µL of the 1ng/µL LCPE Analyte Standard Mix

After combining the two extracts:

To **ALL** Samples:

Add 100µL of the 1ng/µL LCHM Internal Standard

To the **AFTER SPIKE** Samples:

Add 200µL of the 1ng/µL LCHM Analyte Standard Mix

Add 200µL of the 1ng/µL LCPE Analyte Standard Mix