Development of a Liquid Chromatography Tandem-Mass Spectrometry Method for Hormonal Growth Promotants in Beef Cattle

By

MARY MOSBURG

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Approved:

Benjamin Moeller, Chair

Robert Poppenga

Russell Hovey

Committee in Charge

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ABSTRACT

Hormonal growth promotants (HGPs) are a class of pharmaceutical agents commonly administered to beef cattle in the United States to improve growth of the animal, and to improve the desired characteristics of retail cuts of meat. In the United States, a high percentage of beef cattle (>80%) are administered at least one HGP prior to slaughter. Hormonal growth promotants can be separated into two classes, either administered compounds which are not endogenously produced or those which are. The synthetic HGPs approved for use in beef cattle are estradiol benzoate, testosterone propionate, trenbolone acetate, melengestrol acetate, and zeranol as well as their metabolites. The HGPs, which can also be endogenous hormones, are 17β-estradiol and progesterone. There is a concern that low residual concentrations of these compounds may remain in the tissue after slaughter, and consumption of tissues containing these compounds may increase the risk of developing breast cancer. A liquid chromatography-tandem mass spectrometry method was developed and validated to detect the low-level presence of HGPs in retail cuts of meat. Validation parameters including accuracy, precision, recovery, matrix effects, limit of detection, limit of quantitation, linear range, and stability were determined. The limits of detection were in the range of 0.1 – 1.0 pg/mg with adequate accuracy and precision. The data suggests the method is fit-for-use to detect HGPs in retail cuts of meat.

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1. Introduction

Exogenous chemicals formulated as growth promotants were first introduced for use in beef cattle during the 1950s, with the first steroidal implant containing estradiol benzoate and progesterone used in 1956 [1-3]. These synthetic compounds were designed to mimic the powerful effects of the endogenous steroidal hormones estrogen, progesterone, and testosterone [2]. The endogenous hormones act as highly specific and potent ligands for intracellular hormone receptors that are present in various tissues distributed throughout the body, to ultimately result in changes in transcriptional regulation of various genes [4]. The circulating concentrations of endogenous steroidal hormones vary in beef cattle due to a number of factors including age, sex, diet, exercise, and in females, certain stages of the estrous cycle and pregnancy [3].

There are several reasons why HGPs are administered to beef cattle, such as for increasing growth rate, ability to gain weight and lessening of unwanted behavioral characteristics. However, HGPs decrease the palatability of meat to consumers by decreasing the marbling scores and tenderness of the end product [5,6]. Male beef cattle are frequently castrated at approximately 6-8 months of age to prevent unwanted behaviors; however, this may lead to meat that is dark, dry, and firm [7]. To help make up for the loss of endogenous hormones due to castration, beef cattle in the United States are administered steroidal based HGPs. Females are usually fed with melengestrol acetate to prevent estrus related behaviors, as females are not commonly spayed [7]. Palatability, including flavor, juiciness, tenderness, and marbling of beef products is important to the marketability of the meat products to consumers. Implanted beef cattle tend to have less marbling and are graded lower quality than the non-implanted beef cattle [8]. The use of HGP implants also results in an increase of ossification and decreases the

marbling score, where the marbling scores decreased by ~15% for heifers and ~10% for steers compared to non-implanted beef cattle [9]. Other studies have shown that the increase of ossification scores is related to the decreased tenderness in the meat. Ossification of bones helps determine the skeletal maturity of a beef carcass. The more mature the carcass, the less palatable the cooked beef [10-12]. In addition, a decrease in marbling is related to a decrease in tenderness and flavor scores. Ultimately, the goal of the beef sector is to have the most efficient production while producing a consistently acceptable quality product [13,14].

Estrogen (estradiol), progesterone, and testosterone are steroidal hormones produced endogenously in mammals. The fact that these hormones are present in mammals may lead to these hormones being present in the tissues, bodily fluids or in products derived from these animals or their byproducts, where the concentration of these hormones can vary due to age, pregnancy status, and physiological status of the animal [2]. Estrogen, progesterone, and testosterone are potent hormones with diverse pharmacological effects. Some of these hormones or similar synthetic agents have been used in humans as therapeutic drugs to treat various conditions or misused by individuals seeking a competitive advantage. While endogenously produced steroidal hormones are essential for reproduction and other processes, exposure to exogenous growth promotants or endogenous agents has been linked to several adverse health effects [15].

The aim of this thesis was to develop and validate a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method sufficiently sensitive to detect intact esters of HGPs in retail cuts of meat at or below the United States Food and Drug Administration (FDA) minimum detection limits. Such a method can then be utilized to screen retail cuts of meat. However, if some of the compounds do not behave as expected based on their structure, there will be the need

for further method development, such as using derivatization strategies to modify the physical chemical structure to increase sample ionization and lower detection limits [16].

2. Background

2.1 Steroidal Hormones

Steroidal hormones are cleared relatively quickly from the systemic circulation [4]. Pharmaceutical companies have utilized structural modifications of the steroid backbone, or dosing formulations, to modify the pharmacokinetic properties of the compounds following administration to humans and animals [4]. The use of subcutaneous implantable slow-release pellets, and esterified formulations of these compounds, allow for long-term release from the dose site into the systemic circulation, reducing the need for multiple administrations to treated animals [17]. Upon release into the systemic circulation, testosterone propionate, estradiol benzoate, trenbolone acetate and melengestrol acetate are distributed into different tissues, metabolized by enzymatic processes, and ultimately excreted, primarily in the urine. The metabolism of these compounds is complex and a mixture of phase I and II metabolites, along with parent compounds, may be present in various biological matrices. The esterified compounds (testosterone propionate, estradiol benzoate, trenbolone acetate and melengestrol acetate) are cleaved by esterases in the blood to form active metabolites, which can bind to specific receptors. The most likely active metabolites for the HGPs are summarized in **Table 1**. Some of the compounds, such as 17β-estradiol, estradiol benzoate, and zeranol will mimic endogenous estrogens, whereas trenbolone acetate and testosterone propionate will mimic endogenous male androgens. Testosterone, testosterone propionate, trenbolone acetate, and their corresponding non-esterified metabolites bind primarily to the androgen receptor [4]. Zeranol, estradiol benzoate, and estradiol bind to the estrogen receptor, while progesterone and melengestrol

acetate bind to the progesterone receptor [18,19]. There is a possibility that metabolites, in addition to the administered parent compounds, may be found in meat [18].

Table 1. FDA approved HGPs for use in livestock animals. The FDA did not set tolerance limits[1] for estradiol benzoate, trenbolone acetate, or zeranol because they metabolize rapidly. The tolerance limit for estradiol can be used for estradiol benzoate.

Compound	Formulation	Primary Active Metabolite	USDA/FDA Tolerance in Matrix (pg/mg)		
			Liver	Fat	Muscle
Estradiol	Ear Implant	Parent	0.24	0.48	0.12
Estradiol benzoate	Ear Implant	Estradiol	0.24	0.48	0.12
Testosterone propionate	Ear Implant	Testosterone	2.6	1.6	0.6
Progesterone	Ear Implant	Parent	15	30	5.0
Trenbolone acetate	Ear Implant	Trenbolone	None set	None set	None set
Zeranol	Ear Implant	Zearalanol	None set	None set	None set
Melengestrol acetate	Feed Additive	Parent	None set	25	None set

2.1.1 Estrogen

Estrogen is an 18-carbon steroid, produced endogenously, which can also be administered as a percutaneous gel, transdermal patch, subcutaneous implant, or orally [20]. The circulating concentrations of estrogen in premenopausal women vary greatly depending on the timing of the menstrual cycle, with premenopausal women having higher concentrations (range 30 – 350 pg/mL) compared to postmenopausal women (~20 pg/mL) [21]. Estrogen is utilized in the female body to mature and maintain the reproductive system. In men, estrogen can help with

[1] The tolerance limit is the concentration of HGP residue in the tissue derived from a food producing animal, below which the tissue can be safely ingested by consumers with no adverse health effects.

their bone maintenance and brain function. Estrogen is an essential hormone for both men and women. However, men require lower concentrations of estrogen than women to function efficiently. While this hormone is beneficial at normal physiological concentrations, it can have adverse effects when concentrations become elevated, and has been linked to several cancers, including those of the breast and endometrial tissues [1,18,19,22]. Cardiovascular problems may also occur in both males and females due to high concentrations of estrogen [23]. Endocrine disrupting chemicals (EDCs) have the potential to cause a significant risk of breast cancer when there is adverse exposure [24]. Breast tissue, cancerous or not, is very sensitive to endogenous hormones or EDCs with about 65% of breast cancers being estrogen receptor positive [22]. Endocrine disrupting chemicals have also been found in food consumed by humans. However, very little is known about the use of hormones in the beef cattle industry [18].

2.1.2 Testosterone

Testosterone is a 19-carbon steroid that occurs endogenously and can also be administered by injection, patch, topical gel, pill, or subcutaneous implant [25]. The concentration of circulating testosterone for non-obese men aged 19-39 years ranged from 2640 to 9160 pg/mL [26]. Testosterone is essential at physiologic concentrations, as it increases bone strength and density. Testosterone also helps protect the heart and coronary arteries from injury or disease and will increase muscle strength. However, adverse effects can occur if the circulating testosterone concentration is higher than the normal range for men and women [27]. Some researchers have suggested that the use of hormonal treatments in beef cattle results in an increased exposure of humans to steroidal hormones, which may lead to an increased risk of negative health-related conditions, including prostate and breast cancer [18,28].

2.2 Growth Promotants Used in Livestock Animals

The beef industry uses HGPs to increase production of muscle while decreasing production costs [29-31]. Hormonal growth promotants may also be used to enhance the flavor and composition of meat, along with modifying undesirable animal behaviors such as aggression, estrus, and sexual activity [32]. The FDA has approved seven HGP products, six of which are administered as ear implants, while one is a feed additive, as shown in **Table 1** above. The HGPs can be formulated as a single agent or in various combinations and are usually suspended in a silicone or cholesterol matrix to prolong their release into the animal's circulation. Livestock producers utilize HGPs for several reasons and the FDA provides withdrawal guidelines for producers to discontinue use prior to slaughter to avoid violative residue concentrations [18]. Maximum tolerance concentrations for residual hormones have been set for various tissues, and residues in excess of these concentrations may occur if the compounds are improperly used, or recommended withdrawal periods are not followed [18]. There is a variation of the concentrations of HGPs present in different tissues of beef cattle. Estradiol, estradiol benzoate, and progesterone can be retained longer and have higher concentrations in fat tissue versus muscle [2]. Some researchers have speculated there may be an accumulation of exogenous hormones in the tissues of these animals, which may produce adverse effects on individuals who consume these meat products [32].

2.2.1 Estradiol and Estradiol Benzoate

Estradiol (**Figure 1**) is a potent hormone that can be both a parent drug and a primary active metabolite of HGP products [18,33]. Estradiol is used to increase the rate of weight gain and feed efficiency in beef cattle on pasture or in feedlots [34]. Feed efficiency is defined as the

units of feed consumed divided by the units of animal weight gain [35]. Each estradiol implant consists of a silicone rubber implant, containing 25.7 mg of estradiol, and is coated with no less than 0.5 mg of oxytetracycline powder, as a local antibacterial, to provide an effective daily dose for at least 200 days [34]. Estradiol is rapidly cleared in the systemic circulation and thus longer-acting esterified formulations have been developed to improve the pharmacokinetics from a depot formulation. Estradiol benzoate (**Figure 1**) is one form of estradiol approved for use in beef cattle in various formulations. Following release into the systemic circulation, esterified compounds can be cleaved via blood esterases to their non-esterified versions, which will have similar pharmacokinetics and pharmacodynamics as were observed following administration of the non-esterified versions [33]. Estradiol benzoate is metabolized to free estradiol and behaves identically to endogenous estrogen, once in the systemic circulation. Therefore, estradiol benzoate will have the same effect in the body by binding to the estrogen receptor.

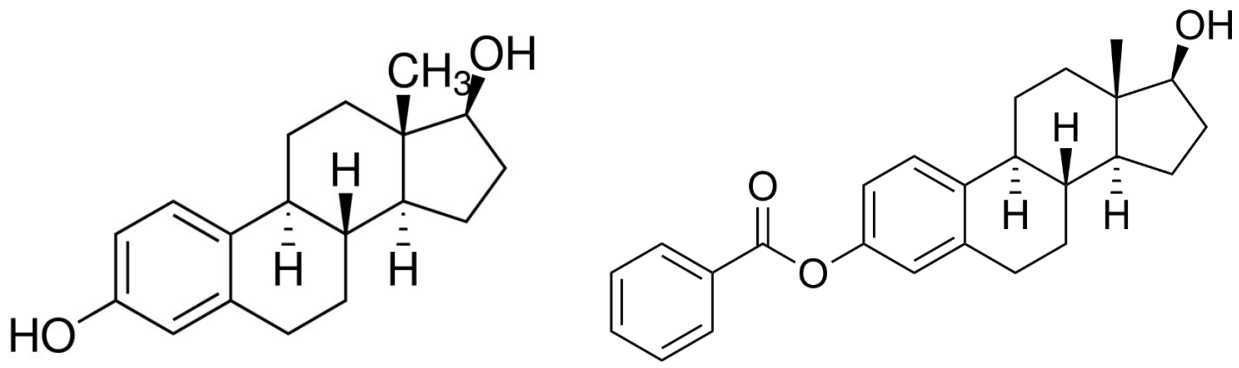


Figure 1. Chemical structures for estradiol and estradiol benzoate

2.2.2 Testosterone Propionate and Testosterone

Testosterone propionate (**Figure 2**) is sold as a slow-release depot formulation with a half-life of around 4.5 days. Testosterone was initially developed in 1974 as an anabolic steroid to increase muscle mass. Testosterone propionate is an ester which can be hydrolyzed into free testosterone (**Figure 2**). Testosterone utilizes several different mechanisms to impart its physiological effects, with two primary pathways believed to have the largest effects. The first pathway follows activation of the androgen receptor, and the second occurs after activation of the estrogen receptor following metabolism by P450 aromatase into estradiol [4]. There are a number of metabolism pathways for testosterone, with estradiol and dihydrotestosterone being two of the active metabolites [33].

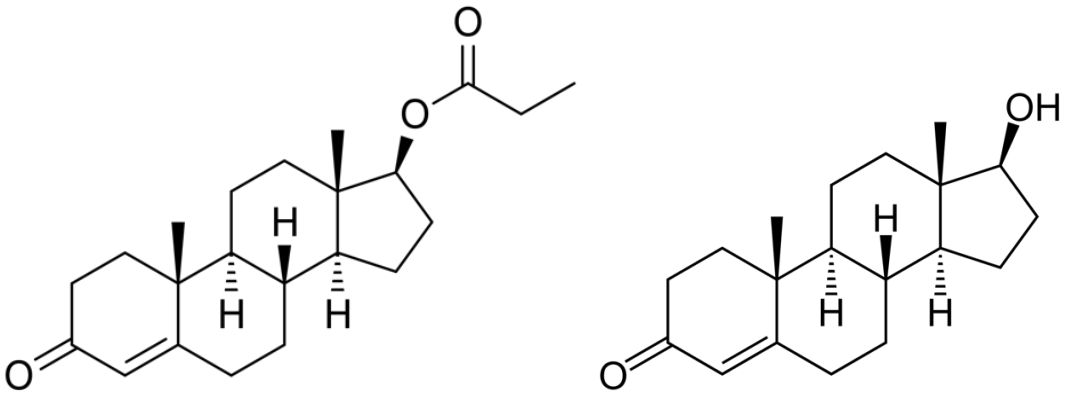


Figure 2. Chemical structures for testosterone propionate and testosterone

2.2.3 Estradiol Benzoate Implant Formulations

Estradiol benzoate and testosterone propionate are typically formulated together for HGPs, but they have different actions. Both are cleaved into their non-esterified versions and circulate throughout the body, where they are then metabolized and ultimately excreted. There

are currently four different formulations of estradiol benzoate and testosterone propionate approved for pharmaceutical use as ear implants in beef cattle. One of the formulations of a single implant contains eight pellets, each comprised of 2.5 mg estradiol benzoate and 25 mg testosterone propionate. This implant is used to help improve the growth rate and feed efficiency in heifers weighing 181 kg or more. There is no withdrawal period established for the estradiol benzoate and testosterone propionate implant [36]. Estradiol benzoate can also be formulated with progesterone (**Figure 3**). Each implant dose for this combination has 20 mg of estradiol benzoate and 200 mg of progesterone with eight pellets each containing 2.5 mg estradiol benzoate and 25 mg progesterone. The estradiol benzoate and progesterone implant is used to increase the rate of weight gain and improve feed efficiency in steers weighing 181 kg or more [37].

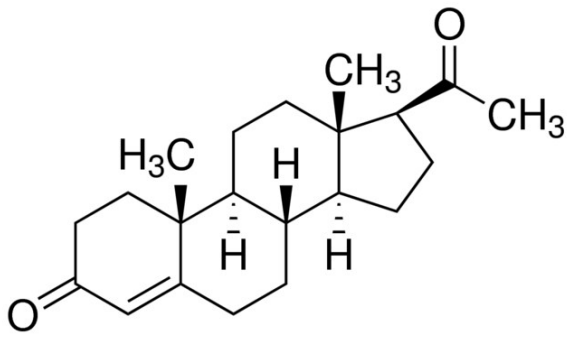


Figure 3. Chemical structure for progesterone

2.2.4 Trenbolone Acetate Implant Formulation

Trenbolone acetate (**Figure 4**) is a slow-release acetate ester, which binds to the androgen receptor, with a half-life of about three days, and is metabolized into trenbolone (**Figure 4**) [38]. In beef cattle, trenbolone acetate is metabolized into the most active form, which is 17β-

trenbolone, and then further metabolized into 17α-trenbolone [18]. Trenbolone acetate is approved for pharmaceutical use in beef cattle with 11 different formulations [38]. One formulation pairs trenbolone acetate with estradiol, as an ear implant consisting of 10 small yellow pellets coated in a polymer for a delayed and extended release. Each pellet consists of active and non-medicinal ingredients. In a single implant, the active ingredients are 200 mg of trenbolone acetate and 20 mg of estradiol. The implant is used to help increase the rate of weight gain and to improve feed efficiency in steers and heifers fed in confinement, which improves grazing performance, for up to 130 days after the feedlot steers and heifers are implanted. When this implant is used, there is no withdrawal period required in beef cattle. However, the implant site should not be kept for use as human or animal food [39].

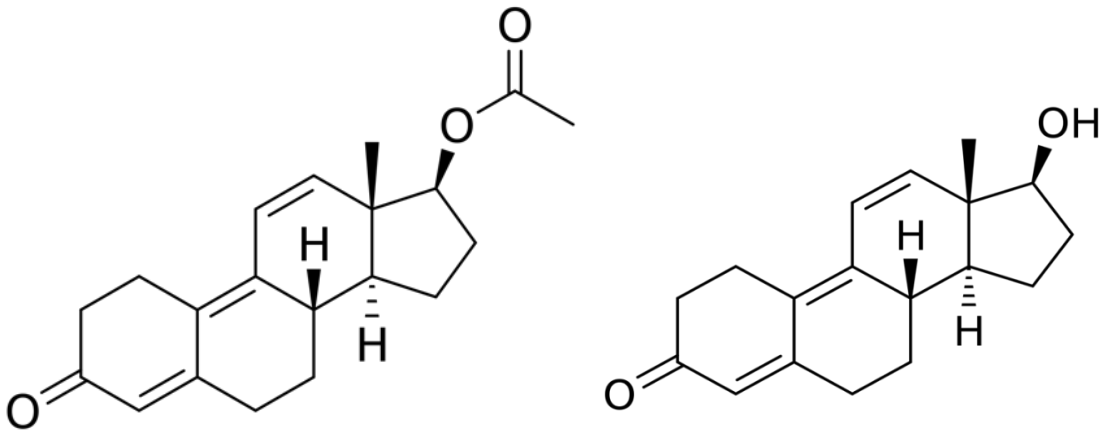


Figure 4. Chemical structures for trenbolone acetate and trenbolone

2.2.5 Zeranol and Zearalanol

Zeranol (**Figure 5**) is an anabolic agent implanted to increase the rate of weight gain and improve feed efficiency of weaned beef calves, growing beef cattle, feedlot steers, and feedlot

heifers. Zeranol also increases the rate of weight gain in suckling beef calves. Each dose contains three small pellets with 12 mg of zeranol in each pellet, delivering a total dose of 36 mg of zeranol. The dose is the same for heifers, steers, and calves. Effective concentrations are reached within the first day following implantation [40]. Similar to trenbolone acetate, there is no tolerance limit set by United States regulatory agencies for zeranol in animal tissue (**Table 1**). Most studies examining residues only focus on zeranol, however zeranol does metabolize into multiple compounds including α- and β-zearalenol, and α- and β-zearalanol (**Figure 5**) [18].

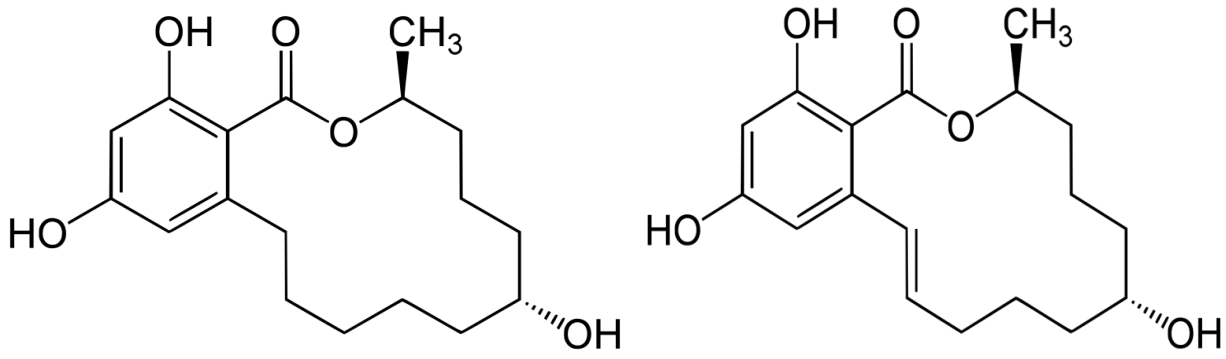


Figure 5. Chemical structures for zeranol and zearalanol

2.2.6 Melengestrol Acetate and Melengestrol

Melengestrol acetate (**Figure 6**) is a synthetic progestogen which becomes active after oral administration. Currently, there are 19 formulations of melengestrol acetate approved for use as pharmaceutical feed additives. Heifers receive melengestrol acetate as a feed additive to increase their weight, improve feed efficiency, and to suppress estrus. Heifers will each be fed between 0.23 kg to 0.90 kg of feed per day to receive 0.25 mg to 0.50 mg of melengestrol acetate (**Figure 6**), which will produce a moderate degree of estrus suppression. To reach a high degree

of estrus suppression, the range of melengestrol acetate should be 0.35 mg to 0.50 mg of daily intake. Lower concentrations can still be effective but provide a lower degree of estrus suppression. There is a withdrawal period of 48 hours prior to slaughter when melengestrol acetate feed is used for heifers. Residues of parent melengestrol acetate containing less than 25 pg/mg in treated animals will not cause a hormonal response. As a result, the tolerance limit of the parent compound melengestrol acetate is 25 pg/mg in the edible tissues of treated animals [41].

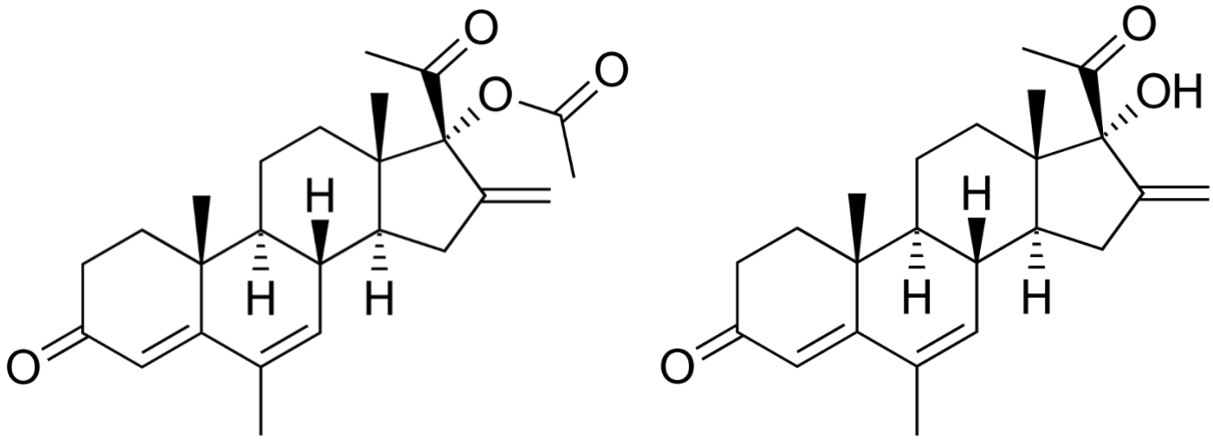


Figure 6. Chemical structures for melengestrol acetate and melengestrol

2.3 Analytical Detection Methodologies

There are several analytical approaches used to detect HGPs, such as liquid chromatography-mass spectrometry (LC-MS), gas chromatography-mass spectrometry (GC-MS), and enzyme-linked immunosorbent assay (ELISA), each with their own strengths and limitations. The largest limitation of ELISA is a high degree of cross-reactivity and therefore less specificity, as compared to MS-based approaches, making it less than ideal for HGP testing [42]. GC-MS has been used extensively to conduct these types of analyses, but with the advent of

more sensitive LC-MS/MS platforms, GC-MS has become less favored [43]. LC-MS/MS has increased in popularity due to its high selectivity and its decrease in interferences, allowing for highly sensitive methods using positive and negative electrospray ionization (ESI) modes [44,45]. Others have found success looking for testosterone esters in meat, including testosterone propionate, using LC-MS though this approach only targeted the esterified versions of the HGPs [44]. Costain and colleagues effectively used the quick, easy, cheap, effective, rugged, and safe (QuEChERS) method to extract their sample before using LC-MS/MS based analysis. The validated method can determine and confirm the presence of exogenous hormones in beef cattle [44].

2.4 Advancing Research

It has been proposed that treating beef cattle with HGPs may pose an increased risk for several adverse health outcomes in consumers who regularly ingest these products [28]. One such outcome is breast cancer, which is more prevalent with increased exposure to estrogen or related compounds [19]. Since increased risk of developing breast cancer has been linked to hormone exposure, there is concern that the concentrations of HGPs used in the livestock industry may contribute to the risk of cancer, through the consumption of meat [18]. Both plants and animals contain steroidal hormones, but the hormones are structurally different [46].

The United States Department of Agriculture (USDA) Food Safety Inspection Service National Residue Program tests for drug residues in animal products and has established tolerance limits as shown in **Table 1** [18]. However, the testing is frequently performed on tissues not commonly consumed by humans, such as the kidney and liver, and chemical residues

are seldom detected [18,47]. Given available data, the FDA and USDA maintain that the HGPs approved for use in beef cattle pose no public health risk when used properly [48].

Many European countries prohibited the use of HGPs in food animals in the late 1980s and subsequently banned the importation of meat from treated animals thereafter, in support of the precautionary principle, to protect consumers before there is scientific proof of a risk [48]. The potential presence of HGPs in consumer meat has resulted in positive drug tests for professional athletes, which illustrates the potential for consumers to inadvertently ingest HGPs and therefore may encounter adverse impacts on their health [49,50].

3. Materials and Methods

3.1 Chemicals and Reagents

17α-estradiol, 17β-estradiol, testosterone propionate, and trenbolone acetate were purchased from Steraloids (Newport, Rhode Island, USA). Estradiol benzoate was purchased from Cayman Chemical Company (Ann Arbor, Michigan, USA). Melengestrol was purchased from Toronto Research Chemicals (North York, Ontario, CA). Melengestrol acetate and α-zearalanol were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Progesterone, testosterone, and trenbolone were purchased from Cerilliant Corporation (Round Rock, Texas, USA). The internal standard 17β-estradiol-D5 was purchased from Steraloids (Newport, Rhode Island, USA), melengestrol acetate-D3 from Santa Cruz Biotechnology, Inc. (Dallas, Texas, USA), and testosterone-D3 from Cerilliant Corporation (Round Rock, Texas, USA).

Methanol (HPLC grade >99.9%) and water (HPLC grade) were purchased from Honeywell – Burdick & Jackson (Muskegon, Michigan, USA). Methanol (optima grade), hexanes (optima grade), ethyl acetate (optima grade), potassium phosphate monobasic (ACS reagent grade) and sodium chloride (NaCl, ACS reagent grade) were purchased from Fisher Scientific (Pittsburgh, Pennsylvania, USA). Potassium hydroxide (reagent grade) and ammonium fluoride (≥99.99% purity by trace metals method) were purchased from Sigma Aldrich (St. Louis, Missouri, USA), and deionized (DI) water from Nanopure water system by Thermo Fisher Scientific (Waltham, Massachusetts, USA).

3.1.1 Selection of Internal Standards for Target Analytes

Stable isotope-labeled internal standard 17β-estradiol-D5 was used as the internal standard for estradiol, 17β-estradiol, and zearalanol. Melengestrol acetate-D3 was used as the internal standard for melengestrol and melengestrol acetate. Testosterone-D3 was used as the internal standard for epi-testosterone, estradiol benzoate, progesterone, testosterone, testosterone propionate, trenbolone, and trenbolone acetate. The standard addition method was used to evaluate the internal standards. Matrix spiking was used to determine how the internal standard would behave in the matrix since the internal standard was not identical to all of the compounds in the drug screen. Three concentrations of HGPs were spiked into the muscle tissue, namely 0.8 pg/mg, 12.5 pg/mg, and 75 pg/mg.

3.1.2 Glassware

16 x 125 mm glass test tubes with screw caps and glass culture tubes (12 x 75 mm) were purchased from Fisher Scientific (Pittsburgh, Pennsylvania, USA). Autosampler vials with fixed inserts were purchased from Agilent Technologies (Santa Clara, California, USA).

3.2 Preparation of Stock Solutions

3.2.1 Reference and Internal Standard Preparation

Reference standards were prepared in methanol at 1 mg/mL for each target analyte, except for testosterone, trenbolone, and progesterone, which were in acetonitrile. An intermediate working standard solution (WS) was made by combining the reference standards and diluting with acetonitrile to achieve a final concentration of 10 ng/µL. The intermediate WS was diluted with methanol to make five WS for the standard curve, the concentrations were: 0.1,

1, 10, 50, and 100 pg/µL. An internal standard intermediate working solution (IS) was prepared from the compounds at 1 mg/mL by combining and diluting them with methanol to 10 ng/µL. The IS solution concentration used was 50 pg/µL.

3.2.2 Liquid-Liquid Extraction Solutions

A 10 M potassium hydroxide (KOH) solution was prepared in deionized water. A 0.1 M phosphate buffer with a pH 9.5 was prepared in deionized water. Adjusted the pH to 9.5±0.1 with 10 M KOH if needed, then brought to volume with deionized water. A 70:30 (v:v) hexanes:ethyl acetate mixture was prepared and mixed. A 0.2 mM ammonium fluoride solution was prepared fresh weekly in HPLC grade water. An 80:20 (v:v) methanol:0.2 mM ammonium fluoride solution mixture was prepared fresh weekly. A 0.9% saline solution was prepared in deionized water, as needed.

3.3 Negative Control Tissues

Muscle used to generate the calibration curve and quality control samples was obtained from a male Jersey calf, humanely euthanized (aged ~5.5 days), in accordance with a protocol approved by the UC Davis Institutional Animal Care and Use Committee (IACUC). After processing, the tissue was stored frozen (-20°C) in 5 g aliquots in 15 mL conical tubes until use.

3.4 Sample Preparation and Liquid-Liquid Extraction

Prior to extraction, the tissue was thawed at room temperature. Once thawed, 100±5 mg of each sample was weighed and placed into a 7 mL bead ruptor bead beating tube from Omni International (Kennesaw, Georgia, USA). The hard tissue homogenizing tube was pre-filled with

an optimized amount of 2.8 mm ceramic beads. The tubes were then filled with 4 mL of 0.1 M phosphate buffer, pH 9.5 and placed into the Omni Bead Ruptor 24 Bead Mill Homogenizer from Omni International (Kennesaw, Georgia, USA) and homogenized into a liquid suspension. Each sample was transferred to a 16 x 125 mm screw top glass vial using a glass Pasteur pipet. A calibration curve was made using the five WS to achieve a final concentration of 0.1, 0.5, 1, 5, 10, 25, 50, and 100 pg/mg of tissue. An IS solution (50 pg/µL) of 50 µL was added to the tubes. Five mL of 70:30 (v:v) hexanes:ethyl acetate solution was added to all tubes and capped tightly. The tubes were put on a rotorack mixer from Glas-Col™ Apparatus Co. (Terre Haute, Indiana, USA) for ~30 minutes at a speed setting of ~25 arbitrary units. The tubes were then centrifuged in a Sorvall ST40R from Thermo Scientific (Waltham, Massachusetts, USA) at 2300 xg for five minutes at 4°C. The top layer was transferred, avoiding the bilayer, to new 12 x 75 mm tubes using glass Pasteur pipets. Samples were then evaporated to dryness using nitrogen in a TurboVap from Zymark Corporation (Hopkinton, Massachusetts, USA) at ~60±5°C. The dry samples were re-dissolved with 100 µL of 80:20 (v:v) methanol:0.2 mM ammonium fluoride and vortexed using a Multi-Pulse Vortexer from Glas-Col™ Apparatus Co. (Terre Haute, Indiana, USA). Finally, the samples were transferred to auto sampler vials with inserts and were capped.

3.5 Solid Phase Extraction

When using solid phase extraction (SPE), the samples were re-dissolved in 2 mL of ethyl acetate instead of 100 µL of 80:20 (v:v) methanol:0.2 mM ammonium fluoride. Bond Elut™ NH2 500 mg cartridges (columns) purchased from Agilent Technologies (Santa Clara, California, USA) were placed on a Cerex SPE positive pressure manifold from Cera Inc. (Baldwin Park, California, USA). The columns were conditioned with 2 mL of hexanes and the

eluate discarded. The samples were applied to conditioned columns and flow through collected, which was combined with another 2 mL of ethyl acetate that was passed through the columns and evaporated using a Turbovap at ~60±5°C.

3.6 UHPLC-MS/MS Analysis

An ultra-high performance liquid chromatography triple quadrupole-mass spectrometry (UHPLC-MS/MS) system comprising a Thermo Vanquish Duo UHPLC coupled with a Thermo Altis^{TM} TSQ MS from Thermo Scientific (Waltham, Massachusetts, USA) was used to analyze the targeted compounds. The HPLC column was an Agilent Poroshell 120 EC-18 (2.7 µm, 3.0 x 50 mm) with a matching LC guard column from Agilent Technologies (Santa Clara, California, USA) that was held at 25°C. The mobile phase A was 100% methanol while the mobile phase B was 0.2 mM ammonium fluoride in water. A reverse phase gradient was used to separate the compounds as follows: time 0.00 min, 50% A; 2.00 min, 98% A; 8.00 min, 98% A; 10.00 min, 50% A. The flow rate was 400 µL/min for the first 10 minutes and 600 µL/min for the last five minutes, with a total run time of 15 minutes. The MS collected spectra using selected reaction monitoring (SRM) to analyze each compound with precursor and product ions, along with their associated collision energies, as shown in **Table 2**. Positive mode ESI was used for trenbolone, testosterone, epi-testosterone, melengestrol, melengestrol acetate, progesterone, trenbolone acetate, testosterone propionate, estradiol benzoate, testosterone-D3, and melengestrol acetate-D3. Negative ESI was used for zearalanol, 17β-estradiol, estradiol, and 17β-estradiol-D5. The following source conditions were used for the mass spectrometer: spray voltage 3500V (+/-), sheath gas 50 arbitrary unit, aux gas 15 arbitrary unit, sweep gas 2 arbitrary unit, ion transfer tube temperature 350°C, and vaporizer temperature of 400°C. The Q1 and Q3 resolutions were

set at 0.7 full width at half-maximum height. The MS was calibrated using Pierce™ extended mass range calibration solution purchased from Thermo Scientific (Waltham, Massachusetts, USA) consisting of a mixture of LC-MS grade acetic acid, imidazole, triethylamine, trifluoroacetic acid, tetramethylpiperidine, 1,8-bis(dimethylamino)naphthalene, 2,4,6-tris(trifluoromethyl)-1,3,5-triazine, hexamethoxyphosphazene, 2,4,6-tris(heptafluoropropyl)-1,3,5-triazine, hexakis(2,2-difluoroethoxy)phosphazene, hexakis(2,2,3,3-tetrafluoropropoxy)phosphazene, hexakis(1h,1h,5h-octafluoropentoxy)phosphazene, hexakis(1h,1h,7h-perfluoroheptoxy)phosphazene, and hexakis(1h,1h,9h-perfluorononyloxy)phosphazene in LC-MS grade acetonitrile, LC-MS grade water, and LC-MS grade 2-propanol (92:4:4 v:v). The LC-MS system was controlled using the Xcalibur™ (version 4.3) and Aria (version 2.6.13) software from Thermo Scientific (Waltham, Massachusetts, USA). Data review was accomplished using Quan Browser software (version 4.3) from Thermo Scientific (Waltham, Massachusetts, USA).

Table 2. LC-MS method settings. Retention time (min), electrospray ionization mode (+/-), radio frequency (RF) voltage (V), SRM transitions showing the precursor and product ion (m/z) with the confirmation ion bolded, and collision energy (eV) of targeted compounds.

Analyte	Retention Time (min)	Positive/Negative ESI Mode	RF (V)	SRM Transition (m/z)	Collision Energy (eV)
17β- Estradiol	3.76	Negative	102	271.2 > **145.0**	39
				271.2 > 183.1	39
				271.2 > 239.2	38
Estradiol	3.97	Negative	102	271.2 > **145.0**	39
				271.2 > 183.1	39
				271.2 > 239.2	38
Zearalanol	3.75	Negative	87	321.2 > 259.1	23
				321.2 > **277.2**	20
				321.2 > 303.2	20
Epi-testosterone	4.53	Positive	58	289.2 > **97.0**	22
				289.2 > 109.0	25
				289.2 > 253.2	17
Estradiol benzoate	6.64	Positive	63	377.2 > **105.0**	21
				377.2 > 135.1	16
				377.2 > 359.2	12
Melengestrol	4.26	Positive	64	355.2 > 236.2	28
				355.2 > **279.1**	21
				355.2 > 337.2	16
Melengestrol acetate	4.92	Positive	60	397.2 > 236.1	29
				397.2 > 279.1	20
				397.2 > **337.2**	14
Progesterone	4.96	Positive	60	315.2 > 97.0	22
				315.2 > 109.0	25
				315.2 > 297.2	16
Testosterone	3.93	Positive	58	289.2 > **97.0**	22
				289.2 > 109.0	25
				289.2 > 253.2	17

Table 2. Continued

Analyte	Retention Time (min)	Positive/Negative ESI Mode	RF (V)	SRM Transition (m/z)	Collision Energy (eV)
Testosterone propionate	6.04	Positive	61	345.2 > **97.0**	23
				345.2 > 109.0	27
				345.2 > 253.1	17
Trenbolone	3.11	Positive	71	271.1 > 199.1	24
				271.1 > 227.1	23
				271.1 > **253.1**	20
Trenbolone acetate	4.83	Positive	71	313.2 > 199.1	28
				313.2 > **253.1**	21
				313.2 > 271.1	19
17β-Estradiol-D5	3.73	Negative	105	276.2 > 145.1	54
				276.2 > 147.0	39
				276.2 > **187.0**	41
Melengestrol acetate-D3	4.91	Positive	60	400.3 > 236.2	29
				400.3 > 279.1	20
				400.3 > **337.2**	14
Testosterone-D3	3.92	Positive	59	292.2 > **97.0**	23
				292.2 > 109.0	26
				292.2 > 274.1	17

3.7 Method Validation

The method was validated as a fit-for-purpose method to detect HGPs in bovine tissues. The following parameters were monitored: accuracy, precision, recovery, matrix effects, stability, carryover, linear range, limit of detection, limit of quantitation, and qualitative identification, per the Association of Official Racing Chemists (AORC) criteria [51].

Seven HGPs and their esters were analyzed, for a total of 12 compounds. Three stable isotope-labeled internal standards (17β-estradiol-D5, melengestrol acetate-D3, and testosterone-

D3) were used to account for analyte loss during sample preparation and extraction. Eight calibration standards containing estradiol, 17β-estradiol, estradiol benzoate, melengestrol, melengestrol acetate, progesterone, testosterone, epi-testosterone, testosterone propionate, trenbolone, trenbolone acetate, and zearalanol were prepared in negative control muscle tissue ranging from 0.1, 0.5, 1, 5, 10, 25, 50 to 100 pg/mg, with an addition of 25 pg/mg of internal standard. Quality control (QC) samples were prepared at low, medium, and high concentrations 0.8, 12.5 and 75 pg/mg, respectively.

To assess accuracy (% accuracy) and precision (relative standard deviation), negative control muscle tissue was spiked with the target analytes at each QC concentration, with six replicate samples, and was evaluated across three days. The average concentration for each QC level was calculated daily. The accuracy was calculated by dividing the average of the QC level by the true value and multiplying by 100 to get a percentage. The inter-day accuracy was calculated by taking the average of the concentration determined each day and dividing it by the true value. The intra-day precision was calculated with the coefficient of variation (%CV) for each target analyte at each QC concentration. The inter-day precision was calculated using the average of the %CV for all three days for all QC levels.

Recovery and matrix effects were evaluated by comparing the instrument response for targeted compounds in extracted samples, including pre-extraction spiked negative control tissue, post-extraction spiked negative control tissue, and neat standards at the equivalent QC samples at low, medium, and high with six replicates per level. Percent recovery was analyzed by dividing the average peak area of the pre-extraction spike by the peak area of the post-extraction spike samples at the three QC concentrations (n=6 replicates/level). Pre-extraction spike samples were spiked with each analyte and the IS before extraction. Post-extraction spike samples were spiked

with each analyte and IS after extraction. Matrix effects were analyzed by dividing the peak area of the QC samples that had been spiked with analytes and IS post-extraction, to six replicate neat standards at each QC concentration.

Spiked stability samples (n=3/day) were evaluated using muscle tissue (100±5 mg) fortified with the targeted compounds at the mid QC concentration and placed in 16 x 125 mm screw top glass vials, followed by the addition of 100 µL of 0.9% saline to ensure complete distribution of the liquid across the tissues. The storage conditions evaluated were room temperature (RT), refrigerator (2-8°C), freezer (-20°C), and two freeze/thaw cycles (-20°C) across various lengths of storage, as shown in **Table 3**. After storage, samples (n=3/condition) were spiked with IS prior to extraction with the other samples, along with a calibration curve. The initial concentration determined on day zero was used as a reference value to analyze the stability samples, where the average concentration on each day analyzed was divided by the average concentration on day zero. Carryover was monitored by using a solvent blank after the high QC sample.

Table 3. Stability conditions including days stored and storage state. The freeze/thaw samples were subjected to two freeze/thaw cycles.

Day	Days Stored	State
1	24 Hours	Room Temperature
		2-8°C
		-20°C
2	48 Hours	2-8°C
		-20°C
3	7 Days	2-8°C
		-20°C
		Freeze/Thaw
4	14 Days	2-8°C
		-20°C
5	21 Days	2-8°C
		-20°C
6	28 Days	2-8°C
		-20°C

Linear range was assessed using negative control muscle tissue samples containing the targeted analytes, followed by linear regression analysis, using the peak area ratio of the targeted compound and its corresponding IS. The peak areas of the IS and targeted compounds were used to calculate the relative response of the quantitation ion. The relative response was calculated by dividing the peak area of the analyte by the peak area of the IS. A linear calibration curve was generated from 0.1 pg/mg to 100 pg/mg of targeted compounds in tissue, to obtain the relative response values for known concentrations, which allowed for linear regression analysis.

The limit of detection (LOD) and limit of quantitation (LOQ) were determined using a signal to noise ratio of 3:1 and 6:1 respectively, from baseline to peak height. The LOD also needed to meet the criteria for the AORC identification. The LOQ had to follow additional criteria where it needed to have a 20% accuracy, relative to the nominal concentration of either 0.8, 12.5, or 75 pg/mg.

4. Results

4.1 Optimization of Instrumental Parameters

The instrumental parameters were optimized to provide the highest signal for the targeted analytes, while providing high selectivity. Mass spectrometer conditions such as collision energy, spray voltage, ESI polarity, precursor ion, product ion, and radio frequency (RF) were identified for each analyte using Thermo Xcalibur™ tuning software, following introduction to the system via an infusion of the compounds (10 ng/µL) with 0.4 mL/min mobile phase flow rate. The RF voltage was optimized for each compound and is shown in **Table 2**. Negative mode ESI was used for ionization of 17β-estradiol, estradiol, zearalanol, and 17β-estradiol-D5 into the MS, while all other compounds utilized positive mode ESI. To determine the retention time, neat standards were run using predetermined LC mobile phase composition and flow rate. Analytes with the same molecular weight needed to be infused separately, as the MS settings could not differentiate between isobaric compounds, therefore chromatographic separation was needed. Tissue samples were then spiked to a final concentration ranging from 0.1 pg/mg to 100 pg/mg to observe how the compounds behaved in matrix and to analyze the extraction procedure efficacy. The most abundant ion was the one selected for the instrument to analyze, so the instrument could achieve the highest sensitivity.

4.2 Extraction Procedures

During the initial method development, tissue samples were either extracted using both liquid-liquid extraction (LLE) with 70:30 hexanes:ethyl acetate and SPE utilizing Bond Elut™ NH2 SPE cartridges or using LLE alone. Following both extractions per sample, the fractions

were analyzed using LC-MS. The results of this approach showed that the use of the amine SPE cartridge resulted in the loss of estradiol, 17β-estradiol, and zearalanol (data not shown), which were present in the extracts of samples only extracted using LLE.

4.3 Method Performance

The linear regression correlation coefficient, R^2, was greater than 0.99 for all analytes with representative results shown in **Table 4**. Accuracy and precision were determined for all analytes by calculating and evaluating inter-day and intra-day statistics as shown in **Table 5**. The intra-day accuracies for QC low ranges were from 84.9% to 108%, QC mid ranges were from 94.6% to 110%, and QC high ranges were from 90.9% to 120%. The inter-day accuracies ranged from 82.9% to 106%, 94.4% to 111%, and 94.5% to 113%, for low, medium, and high QC concentrations, respectively. Depending on the analyte, the inter-day precision ranged from 6.32% to 54.2%, 4.72% to 20.6%, and 3.57% to 20.6%, for low, medium, and high QC concentrations, respectively. The average recovery for the IS 17β-estradiol-D5, melengestrol acetate-D3, and testosterone-D3 was 84.4%, 88.6%, and 87.6%, respectively. Percent recovery, matrix effects, LOD, and LOQ are shown in **Table 6**. To assess the stability of the analytes in tissue samples, negative control muscle tissue was soaked in a spiked concentration of 12.5 pg/mg and 0.9% NaCl solution and stored at pre-defined storage conditions for various times. **Table 7** shows the accuracy for the stability samples. **Figure 7** shows the extracted chromatographic peaks with retention times at the mid QC concentration, 12.5 pg/mg.

Table 4. Representative validation results for linear regression analysis, including intercept, slope, and regression coefficient (R^2).

Analyte	Linearity		
	Intercept	**Slope**	**R²**
17β-Estradiol	0.00111	0.0542	0.999
Estradiol	-0.00225	0.0972	0.998
Epi-testosterone	0.00241	0.0190	0.999
Estradiol benzoate	0.000198	0.00431	0.993
Melengestrol	0.0000271	0.0171	0.999
Melengestrol acetate	0.00234	0.0380	0.999
Progesterone	0.00904	0.0344	0.999
Testosterone	0.000257	0.0378	0.999
Testosterone propionate	-0.000718	0.0180	0.999
Trenbolone	0.000433	0.0314	0.999
Trenbolone acetate	0.000109	0.108	0.998
Zearalanol	-0.00359	0.437	0.999

Table 5. Validation results for inter-day and intra-day accuracy and precision for low, mid, and high QC concentrations. The QC concentrations were 0.8 pg/mg, 12.5 pg/mg, and 75 pg/mg, respectively.

Analyte	Nominal Concentration (pg/mg)	Day 1 Accuracy (%)	Day 1 Precision (%)	Day 2 Accuracy (%)	Day 2 Precision (%)	Day 3 Accuracy (%)	Day 3 Precision (%)	Inter-day Accuracy (%)	Inter-day Precision (%)
17β-Estradiol	0.8	99.4	65.5	92.6	13.9	84.9	24.5	106	54.2
	12.5	97.6	3.76	94.4	6.12	94.6	4.03	95.5	4.72
	75	140	4.12	91.2	2.33	103	3.94	98.0	6.35
Estradiol	0.8	95.7	7.54	87.7	8.14	104	10.2	95.9	11.1
	12.5	97.6	3.85	97.4	7.68	104	6.33	99.7	6.63
	75	106	3.31	102	1.77	120	3.43	109	7.71
Zearalanol	0.8	93.7	3.94	85.6	3.69	97.9	9.33	92.4	8.31
	12.5	103	6.22	95.4	5.55	104	4.57	101	6.47
	75	99.5	3.27	90.9	2.42	108	3.54	99.5	7.78
Epi-testosterone	0.8	113	4.43	92.9	4.73	104	12.5	103	11.2
	12.5	113	3.15	90.6	5.66	105	5.26	103	10.1
	75	109	3.35	87.1	4.01	104	3.99	100	10.3
Estradiol benzoate	0.8	85.7	17.7	105	33.4	102	11.4	97.8	24.0
	12.5	102	11.2	91.9	18.9	93	7.31	95.5	13.4
	75	97.0	19.6	101	11.0	97	16.1	98.4	15.0
Melengestrol	0.8	101	7.81	110	6.13	94.1	10.7	102	10.2
	12.5	99.4	6.47	123	5.72	110	4.96	111	10.5
	75	97.6	5.27	123	2.43	118	3.38	113	10.7
Melengestrol acetate	0.8	90.0	4.03	94.9	3.07	99.5	6.86	94.8	6.32
	12.5	100	3.13	100	4.84	104	3.83	101	4.26
	75	99.1	2.38	97.9	3.52	108	2.23	102	5.29
Progesterone	0.8	83.7	7.85	72.9	18.8	92.2	13.3	82.9	16.1
	12.5	103	5.68	85.5	7.03	100	4.40	96.4	9.87
	75	106	4.35	82.0	3.80	95.9	3.62	94.5	11.2
Testosterone	0.8	92.0	3.04	89.9	1.75	97.1	8.91	93.0	6.35
	12.5	99.9	1.54	102	4.79	103	4.38	102	3.92
	75	98.1	2.37	101	2.35	105	1.53	101	3.57
Testosterone propionate	0.8	103	6.08	84.4	9.86	100	9.65	95.9	12.0
	12.5	109	5.35	82.8	6.66	91.6	7.00	94.4	13.1
	75	117	6.06	85.0	5.62	95.1	8.98	98.9	15.2
Trenbolone	0.8	90.5	6.30	103	19.3	89.8	11.5	94.4	14.8
	12.5	98.7	6.15	108	4.17	95.5	7.44	101	7.79
	75	96.2	7.84	104	4.46	90.9	10.3	97.0	9.15
Trenbolone acetate	0.8	102	8.95	86.0	9.18	108	7.01	98.8	12.5
	12.5	134	9.40	85.0	12.1	110	6.74	109	20.6
	75	128	14.3	83.0	3.17	104	3.21	105	20.6

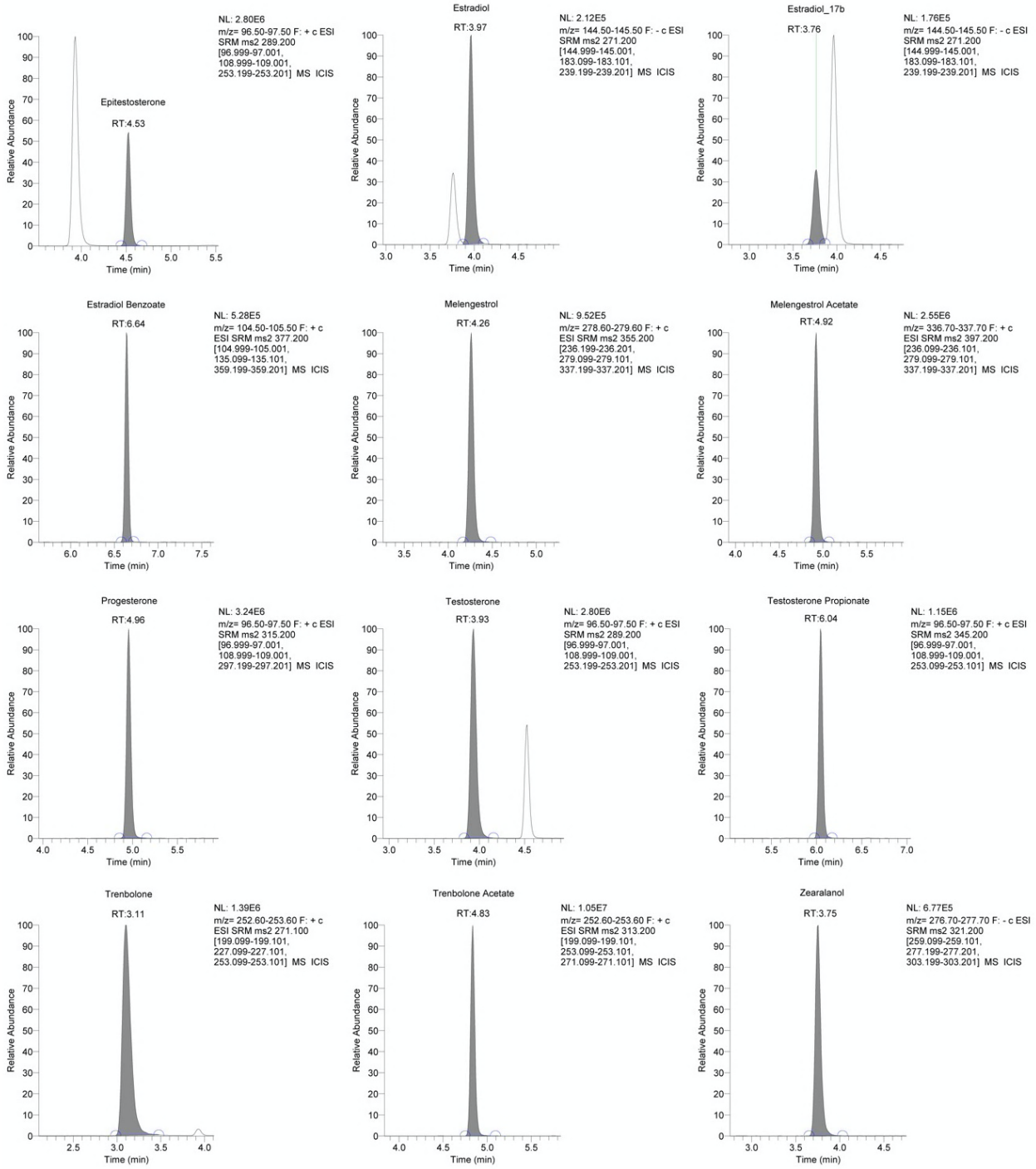
Table 6. Validation results for recovery (%), matrix effects (%), limit of detection (pg/mg), and limit of quantitation (pg/mg).

Analyte	Recovery (%)			Matrix Effect (%)			LOD	LOQ
	0.8 pg/mg	12.5 pg/mg	75 pg/mg	0.8 pg/mg	12.5 pg/mg	75 pg/mg	(pg/mg)	(pg/mg)
17β-Estradiol	108	104	109	45.6	52.6	52.3	0.1	0.5
Estradiol	95.2	93.6	106	99.3	106	99.3	0.1	0.5
Epi-testosterone	92.5	91	96.8	102	109	102	0.1	0.5
Estradiol benzoate	87.2	95.6	70.2	45.1	14	46.8	1	5
Melengestrol	84.2	92.9	90.1	23.2	110	108	0.5	1
Melengestrol acetate	96.8	95	97.1	94.2	102	91.5	0.1	0.5
Progesterone	98.7	96	96.1	132	114	105	0.5	1
Testosterone	96.5	97.3	96.4	90.1	93	98.4	0.1	0.5
Testosterone propionate	94.8	90.4	91.4	82	81.7	87.1	0.1	0.5
Trenbolone	117	112	98.6	217	177	114	0.1	0.5
Trenbolone acetate	74.3	71.7	83.3	352	371	202	0.1	0.5
Zearalanol	93.6	95.9	101	131	127	113	0.1	0.5

Table 7. Stability results of mid QC concentration, 12.5 pg/mg (% Accuracy). Samples were stored at room temperature (RT), refrigerator (4°C), freezer (-20°C), and two freeze/thaw cycles.

Analyte	Stability RT		Stability 4°C						Stability -20°C						
	0.0 hr	24 hr	24 hr	48 hr	7 days	14 days	21 days	28 days	24 hr	48 hr	7 days	14 days	21 days	28 days	Freeze /Thaw 7 days
17β-Estradiol	100	105	111	113	118	133	117	127	98.0	98.0	99.5	91.1	84.8	97.3	82.4
Estradiol	100	119	112	104	107	113.5	129	108	103	90.5	96.3	93.4	117	114	83.3
Epi-testosterone	100	91.0	90.2	89.0	74.8	100	98.4	101	84.6	78.3	69.9	84.2	89.6	105	65
Estradiol benzoate	100	38.8	35.4	36.0	25.5	31.3	38.6	22.2	34.2	27.0	20.2	20.1	37.6	18.6	21.4
Melengestrol	100	126	117	136	129	125	144	110	107	130	123	112	133	116	100
Melengestrol acetate	100	95.4	105	110	97.2	102.4	120	99.9	95.4	104	90	87.2	103	98.1	74.5
Progesterone	100	75.1	84.0	82.2	73.3	85.7	93.7	94.6	76.8	72.6	65.9	69.2	81.2	94.1	62.5
Testosterone	100	102	107	109	104	111	117	107	104	105	96.3	98.1	110	104	88.5
Testosterone propionate	100	61.3	65.0	77.4	63.9	76.6	72.2	64.7	58.1	59.3	58.3	51.9	53.8	69.7	48.1
Trenbolone	100	82.8	88.3	63.0	78.4	100.8	74.8	69.8	85.7	58.7	75.7	92.9	88.4	100.1	70.4
Trenbolone acetate	100	77.1	89.9	103	72.7	71.3	79.5	64.9	84.2	90.0	69.2	64.6	86.5	90	63.9
Zearalanol	100	114	119	130	113	121	127	105	114	118	104	104	113	105	92.3

Figure 7. Extracted ion chromatogram of targeted compounds at mid QC level (12.5 pg/mg).



5. Discussion

The analysis of HGPs has a significant bearing on health outcomes due to potential HGP exposure to humans following their consumption of retail meat products containing HGPs. Determining the exposure of a population to HGPs requires the necessary analytical methodologies to detect the HGP substances in the appropriate matrices which consumers ingest. Previously, some of these HGPs were analyzed in matrices not commonly consumed by humans, such as the kidney and liver [18,47]. Some researchers have hypothesized that treating beef cattle with HGPs causes an increased risk of adverse health outcomes to consumers who regularly ingest products derived from animals treated with HGPs [28]. One of these adverse outcomes is breast cancer. Since an increased risk for breast cancer has been linked to exposure of elevated concentrations of estrogen, there is a concern that HGPs used in livestock may be contributing to breast cancer occurrence [18,19]. The development of this highly sensitive, selective, and high throughput method using UHPLC-MS/MS provides an important quantitative screening tool for potential positive samples. The method was developed as a quantitative method but will be used as qualitative screening to determine if confirmatory analysis is needed.

A handful of studies have tested for residues of HGPs in consumer beef products, though none of the reports focused on the same breadth of compounds as reported here [29,44,52-54]. **Table 8** is a review of literature showing extraction and detection approaches, matrices used, and LOD for various HGPs. Due to the narrow focus of most of the previous studies, this has led to missing some human HGP exposures from the consumption of HGP treated meat [28]. Studies in Taiwan and Austria found that zeranol was not present in beef or bovine urine above the accepted quantitation limits of 25 ng/mL in beef and 2 ng/mL in urine [53,54]. In France, low-

levels of residues for 17α-estradiol (0.06 pg/mg), 17β-estradiol (0.09 pg/mg), testosterone (0.02 pg/mg), and epi-testosterone (0.05 pg/mg) were present in the liver, kidney, and muscle of beef cattle implanted with estradiol and trenbolone [52]. In a Turkish study, meat from markets in Istanbul was frequently positive for zeranol (100% of samples tested) and trenbolone (80% of samples tested) [29].

Table 8. Previous studies summarizing extraction approaches, detection approaches, matrices collected and limits of detection for HGPs.

Compound	Matrix	Extraction Approach	Detection Method	Detection Limit	Citation
Testosterone propionate	Injection site beef tissue	LLE	LC-MS/MS	65 pg/mg	Costain, et al., 2008
Trenbolone acetate	Injection site beef tissue	LLE	LC-MS/MS	18 pg/mg	Costain, et al., 2008
Testosterone	Egg, meat, and milk	LLE	GC-MS/MS	0.01 pg/mg	Courant, et al., 2008
Estradiol	Egg, meat, and milk	LLE	GC-MS/MS	0.01 pg/mg	Courant, et al., 2008
Trenbolone	Bovine meat	LLE	ELISA	0.01 pg/mg	Nazli, et al., 2005
Zeranol	Bovine meat	LLE	ELISA	0.01 pg/mg	Nazli, et al., 2005
Zeranol	Beef, pork, and chicken	SPE	HPLC-EC	25 ng/mL	Hsieh, et al., 2013
Zeranol	Bovine urine	SPE	LC-MS/MS	2 ng/mL	Kleinova, et al., 2002

Different chromatographic conditions (mobile phase composition and temperature) were evaluated to achieve the optimum chromatography and separation (data not shown). The use of an ammonium fluoride mobile phase modifier and a C18 HPLC column allowed for adequate separation and performance, with all compounds eluting between 3.0 and 7.0 minutes, as shown in **Figure 7**. Overall, the esterified versions of the targeted compounds were retained longer on

the HPLC column and resulted in elution approximately 30 seconds to 3 minutes later than their non-esterified free metabolites. In addition to reducing interference, achieving adequate chromatographic separation is important for the compounds that exist as isomers with identical precursor and product ions following MS based fragmentation. For example, 17α-estradiol and 17β-estradiol, along with testosterone and epi-testosterone, differ only on the positioning of the hydroxyl group located at the 17 position on the steroid backbone and produce identical product ion ratios. Accordingly, care was taken to achieve chromatographic baseline separation between the two compounds. For the estradiol isomers, the compounds were separated by 0.2 min, while testosterone and epi-testosterone were separated by 0.6 min. Being able to chromatographically separate the compounds was an ideal outcome as we are now able to identify and differentiate between close eluting compounds in a sample.

During initial method development, a Thermo Q Exactive™ orbitrap MS supplied by Thermo Scientific (Waltham, Massachusetts, USA) with an Agilent HPLC was used to detect the compounds following chromatographic separation in either positive or negative ESI. The instrument utilized full scan capabilities of the selected precursor ion. However, the instrument scan speed was too slow when switching between positive and negative ESI modes.

After evaluating method performance on the Q Exactive system, it was then transferred onto a Thermo Altis™ triple quadrupole MS, due to its higher scan speeds, good sensitivity, and ability to quickly switch between positive and negative ESI modes with minimal impacts. The Thermo Altis™ utilizes an S-Lens, which is a stacked-ring ion design, and it is an RF-only device. The S-Lens efficiently captures and focuses the ions into a tight beam, without needing a DC gradient, to propel them forward. Radio frequency voltages were applied to the series of flat ring electrodes. The orifice diameter of the ring electrodes at the entrance of the S-Lens are

larger, making it capable of capturing as many ions as possible entering the ion transfer tube. A voltage called the delustering potential is applied to the orifice. This helps prevent the ions from clustering together when entering the orifice. The larger orifice increases the LOD and LOQ [55]. After switching to the Altis, direct infusion of neat standards was done with both positive and negative ESI to determine the precursor ions, product ions, collision energy, and RF voltage (**Table 2**). Neat standards were then run through the method to determine the retention times of the target analytes. The retention time aids in the identification of positive samples.

Analyzing steroids with LC-MS can be challenging due to poor ESI based ionization efficiency, because the molecules only rely on either hydroxyl or aldehyde functional groups to carry a charge, which is necessary for detection on the system. All 12 compounds analyzed were nonpolar and lacked a nitrogen in their structures, which commonly enhances ionization efficiency using ESI. Estradiol has a better sensitivity when using negative ESI mode, especially when using ammonium fluoride as a mobile phase modifier, resulting in increased sensitivity, when paired with reversed-phase C18 column and detection using ESI [56,57]. The use of 0.2 mM ammonium fluoride for the mobile phase was critical for this method to work, where others recorded that large increases in the concentration of ammonium fluoride as a modifier result in ion suppression due to elevated presence of negative ions. If the concentration of ammonium fluoride was too low, it resulted in poor ionization [56]. Ammonium fluoride helped with the ionization of underivatized estradiol by enhancing the negative ion electrospray performance eliminating the need for derivatization [56,58].

Some HGP products are formulated to contain synthetic versions of endogenously produced compounds (17β-estradiol, estradiol, progesterone, testosterone, and epi-testosterone) which complicates analysis, as a challenge can be obtaining a blank matrix for quantitative

analysis. During the initial stages of development, ground beef purchased at a local retailer (Davis, California, USA) was used as the blank matrix. The product was labeled as 100% natural, grass fed and 93% lean. This meat, however, contained detectable amounts of progesterone. Various brands and different packages of 93% lean ground beef were also subsequently tested, yet still had detectable amounts of progesterone. Veal was also tested and came up positive for progesterone, but at a lower concentration. The concentrations in the veal were still too high to be considered a good negative control while allowing for low-level quantitation. Accordingly, tissue samples were obtained from a Jersey bull calf (~5.5 days old) to minimize the endogenous presence of the hormones in the tested tissues.

Sample preparation prior to instrumental analysis is important in achieving low detection limits with minimal interference. In the case of tissue analyzed in this study, the use of bead homogenization allowed for a complete homogenization of tissue into an aqueous solution of 0.1 M phosphate buffer at a pH of 9.5. A 70:30 hexanes:ethyl acetate mixture, which is immiscible with water, was added allowing for a phase separation between the aqueous and organic solvents. The hexanes:ethyl acetate mixture extracted compounds which were either uncharged or had a pKA above 9.5. The use of the high pH buffer solution helped reduce background noise by retaining charged compounds or compounds below a pKA of 9.5 [59,60]. Thus, any compounds that were ionizable and had a pKA below 9.5 were not efficiently extracted by the LLE, which reduced the amount of background noise recorded on the MS. Initially, SPE was employed using Bond Elut^{TM} NH2 SPE columns following the LLE, to further reduce the background noise of positively charged species. However, it was determined that this clean-up step was dramatically reducing recovery for estradiol and zearalanol. Further analysis was performed using both the SPE clean-up step and no SPE clean-up step. Each sample had been split into two separate 12 x

75 mm test tubes (A and B) during the LLE step. Sample A went through the SPE clean-up step and sample B was dried down and re-dissolved for analysis. The results determined that the clean-up step was not necessary since it did not provide significantly better peaks (data not shown). Skipping the clean-up step also saved time because the samples did not need to go through the split process.

The accuracy and precision were acceptable for the majority of the compounds, with most accuracies falling between 84.9% to 108%, 94.6% to 110%, and 90.9% to 120%, for low, medium, and high QC concentrations, respectively. The inter-day precision was between 6.32% to 54.2% CV, 4.72% to 20.6% CV, and 3.57% to 20.6% CV for low, medium, and high QC concentrations, respectively, as shown in **Table 5**. However, precision was higher than 20% for both estradiol and estradiol benzoate at the low QC concentration, most likely due to being close to or below their limit of detection. Most compounds had acceptable recoveries (>80%) at 0.8 pg/mg, 12.5 pg/mg, and 75 pg/mg with averages of 96.8%, 96.7%, and 95.7%, respectively. The average recovery for trenbolone acetate was lower, at 76.2%, which is lower than what others have observed, at 87.9% [61].

Matrix effects occur by ion suppression or ion enhancement. If there is ion suppression, then the matrix effect is < 1. If there is ion enhancement, then the matrix effect is > 1. If there is no matrix effect, then it is equal to 1 [62]. The matrix effects we observed ranged from 0.353 to 3.08 in our samples. Ion suppression was observed for five compounds and the average values were: 17β-estradiol, 0.502; estradiol benzoate, 0.353; melengestrol, 0.804; testosterone, 0.938; and testosterone propionate, 0.836. Ion enhancement was observed in six compounds and the average values were: estradiol, 1.02; epi-testosterone, 1.04; progesterone, 1.17; trenbolone, 1.69;

trenbolone acetate, 3.08; and zearalanol, 1.24. There was one compound, melengestrol acetate, with no matrix effects, and the average value was 1.00.

The LOD and LOQ are important parameters to analyze during method development. As shown in **Table 6**, most of the LOD were at or below 0.5 pg/mg, except for estradiol benzoate, which was 1 pg/mg. Many of the compounds in **Table 8** that were analyzed in other studies have been shown to have a higher detection limit than found in our study. Others have successfully detected testosterone and estradiol at a lower limit (0.01 pg/mg) using GC-MS/MS than we detected here [52]. Both estradiol and estradiol benzoate exceeded 20% CV at the lowest QC concentration due to the lowest QC having a concentration at or lower than the LOD for these compounds. The LOD and LOQ were used in our analytical procedure to show the smallest concentration of an analyte that can be accurately measured. The LOD and LOQ were determined by measuring the signal to noise ratio from the baseline to the peak height. To meet the qualitative identification criteria outlined by the AORC, each precursor ion needed to have a minimum of three product ions present with ion ratios of 30% relative or 10% absolute of the corresponding standard [51]. Progesterone was the only compound that failed to meet the strict criteria set by the AORC. The three product ions for progesterone were not observed until 5 pg/mg. However, samples were identified as being positive for levels as low as 0.5 pg/mg, due to strong dominant ions at 97 and 109 m/z.

The IS we used reflected their similarity to the target analytes. By having an IS which behaves similarly to the analyte, then anything that might affect the analyte would also affect the IS. The addition of IS would make the relative response more accurate. 17β-estradiol-D5 was used for 17α-estradiol, 17β-estradiol and zearalanol. Melengestrol acetate- D3 was used for melengestrol and melengestrol acetate. Testosterone-D3 was used for the remaining compounds.

The IS mimicked the chemical properties of their target compounds and allowed for ideal performance of the assay.

The stability of a compound is a particularly important factor in the overall utility of a given approach. In our study, the stability storage conditions were chosen to mimic the ways consumers may store their meat (RT, 4°C, and -20°C). The stability samples underwent the same extraction procedure as described previously. However, the stability samples also had 0.9% saline added to ensure the tissue was fully covered in spiking solution. The samples were run along with a calibration curve. The stability of the samples was then evaluated based on the percent of the initial concentration on day zero. As shown in **Table 7**, most compounds were relatively stable at room temperature; however, the compounds were more stable at both 4°C and -20°C. The best storage condition for preservation of the compounds was deemed to be -20°C. Estradiol benzoate degraded by ~65% and testosterone propionate degraded by ~40% in matrix after 24 hours in all storage conditions. While the exact mechanism for the losses was not determined, it could be due to the non-specific binding of the compounds to the glassware. The freeze/thaw cycle did exacerbate degradation, as opposed to leaving the samples untouched in the 4°C and -20°C storage conditions. Over time, a decrease in analyte response did occur due to the analyte degrading. These results suggest that except for estradiol benzoate, the majority of compounds will likely continue to be found in meat available for consumption if they were initially present.

5.1 Conclusion

A method to detect 12 HGPs was successfully developed and validated using UHPLC-MS/MS. The method was successfully validated to analyze 17β-estradiol, estradiol, epi-

testosterone, estradiol benzoate, melengestrol, melengestrol acetate, progesterone, testosterone, testosterone propionate, trenbolone, trenbolone acetate, and zearalanol in beef tissues, with low limits of detection and quantitation. Liquid-liquid extraction using a high pH allowed for selective removal of charged species and good concentration of extraction prior to instrumental analysis. Validation parameters included accuracy, precision, recovery, matrix effects, stability, carryover, linear range, limit of detection, and limit of quantitation, all showing acceptable results. The compounds in beef were stable during storage at temperature ranges commonly utilized by consumers, with the exception of estradiol benzoate and testosterone propionate which degraded. These findings have determined that if the compounds are present in raw store-bought meat, there is a possibility consumers may be at risk of exposure by ingesting residual HGPs.

REFERENCES

1. Handa, Y., et al., *Estrogen concentrations in beef and human hormone-dependent cancers.* Annals of Oncology, 2009. **20**(9): p. 1610-1611.
2. Waltner-Toews, D. and S.A. McEwen, *Residues of hormonal substances in foods of animal origin: a risk assessment.* Preventive Veterinary Medicine, 1994. **20**(3): p. 235-247.
3. Doyle, E. *Human Safety of Hormone Implants Used to Promote Growth in Cattle*. 2000. 1-24.
4. Kicman, A.T., *Pharmacology of anabolic steroids.* British Journal of Pharmacology, 2008. **154**(3): p. 502-521.
5. *Steroid Hormone Implants Used for Growth in Food-Producing Animals*. Available from: https://www.fda.gov/animal-veterinary/product-safety-information/steroid-hormone-implants-used-growth-food-producing-animals.
6. APHIS. *The Use of Growth-Promoting Implants in U.S. Feedlots.* 2013; USDA-APHIS-VS-CEAH-NAHMS:[Available from: https://www.aphis.usda.gov/animal_health/nahms/feedlot/downloads/feedlot2011/Feed11_is_Implant_1.pdf.
7. Drouillard, J.S., *Current situation and future trends for beef production in the United States of America — A review.* Asian-Australasian Journal of Animal Sciences, 2018. **31**(7): p. 1007-1016.
8. Nichols, W.T., et al., *Effects of Steroid Implants on the Tenderness of Beef.* The Professional Animal Scientist, 2002. **18**(3): p. 202-210.
9. Watson, R., et al., *Effect of hormonal growth promotants on palatability and carcass traits of various muscles from steer and heifer carcasses from a Bos indicus–Bos taurus composite cross.* Australian Journal of Experimental Agriculture, 2008. **48**(11): p. 1415-1424.
10. Smith, G.C., et al., *USDA Maturity Indices And Palatability Of Beef Rib Steaks.* Journal of Food Quality, 1988. **11**(1): p. 1-13.
11. Park, B.Y., et al., *Effect of carcass suspension and cooking method on the palatability of three beef muscles as assessed by Korean and Australian consumers.* Australian Journal of Experimental Agriculture, 2008. **48**(11): p. 1396-1404.
12. Smith, G.C., et al., *Relationship of USDA marbling groups to palatability of cooked beef.* Journal of Food Quality, 1985. **7**(4): p. 289-308.
13. Dikeman, M.E. *The Relationship of Animal Leanness to Meat Tenderness*. in *Reciprocal Meat Conference*. 1996.
14. Thompson, J.M., *The effects of marbling on flavour and juiciness scores of cooked beef, after adjusting to a constant tenderness.* Australian Journal of Experimental Agriculture, 2004. **44**(7): p. 645-652.
15. Feigelson, H.S. and B.E. Henderson, *Estrogens and breast cancer.* Carcinogenesis, 1996. **17**(11): p. 2279-2284.
16. Marcos, J. and O.J. Pozo, *Derivatization of steroids in biological samples for GC–MS and LC–MS analyses.* Bioanalysis, 2015. **7**(19): p. 2515-2536.

17. Smith, Z.K., et al., *Effects of a single initial and delayed release implant on arrival compared with a non-coated initial implant and a non-coated terminal implant in heifers fed across various days on feed.* Translational Animal Science, 2019. **3**(4): p. 1182-1193.
18. Nachman, K.E. and T.J.S. Smith, *Hormone Use in Food Animal Production: Assessing Potential Dietary Exposures and Breast Cancer Risk.* Current Environmental Health Reports, 2015. **2**(1): p. 1-14.
19. Yager, J.D. and N.E. Davidson, *Estrogen Carcinogenesis in Breast Cancer.* New England Journal of Medicine, 2006. **354**(3): p. 270-282.
20. Stevenson, J.C., *Type and route of estrogen administration.* Climacteric, 2009. **12**(sup1): p. 86-90.
21. Kuhl, H., *Pharmacology of estrogens and progestogens: influence of different routes of administration.* Climacteric, 2005. **8**(sup1): p. 3-63.
22. Lecomte, S., et al., *Emerging Estrogenic Pollutants in the Aquatic Environment and Breast Cancer.* Genes, 2017. **8**(9): p. 229.
23. Nelson, L.R. and S.E. Bulun, *Estrogen production and action.* Journal of the American Academy of Dermatology, 2001. **45**(3): p. S116-S124.
24. Vandenberg, L.N., et al., *Hormones and Endocrine-Disrupting Chemicals: Low-Dose Effects and Nonmonotonic Dose Responses.* Endocrine Reviews, 2012. **33**(3): p. 378-455.
25. Margo, K.L. and R. Winn, *Testosterone Treatments: Why, When, and How?* American Family Physician, 2006. **73**(9): p. 1591-1598.
26. Travison, T.G., et al., *Harmonized Reference Ranges for Circulating Testosterone Levels in Men of Four Cohort Studies in the United States and Europe.* The Journal of Clinical Endocrinology & Metabolism, 2017. **102**(4): p. 1161-1173.
27. Osterberg, E.C., A.M. Bernie, and R. Ramasamy, *Risks of testosterone replacement therapy in men.* Indian Journal of Urology: IJU: Journal of the Urological Society of India, 2014. **30**(1): p. 2-7.
28. Andersson, A.M. and N.E. Skakkebaek, *Exposure to exogenous estrogens in food: possible impact on human development and health.* European Journal of Endocrinology Eur J Endocrinol, 1999. **140**(6): p. 477-485.
29. Nazli, B., et al., *The Presence of Some Anabolic Residues in Meat and Meat Products Sold in Istanbul.* Turkish Journal of Veterinary and Animal Sciences, 2005. **29**(3): p. 691-699.
30. Ward, A.S., et al., *Coupled reversion and stream-hyporheic exchange processes increase environmental persistence of trenbolone metabolites.* Nature Communications, 2015. **6**(1): p. 7067.
31. Stephany, R.W., *Hormonal Growth Promoting Agents in Food Producing Animals*, in *Doping in Sports: Biochemical Principles, Effects and Analysis*. 2010, Springer Berlin Heidelberg: Berlin, Heidelberg. p. 355-367.
32. Joseph, S., et al., *An End-to-End Workflow Solution For Quick and Easy Quantitative Analysis of Multiclass Veterinary Drug Residues in Meat Using LC-MS/MS.* p. 1.
33. Wishart, D.S., et al., *DrugBank 5.0: a major update to the DrugBank database for 2018.* Nucleic Acids Research, 2017. **46**(D1): p. D1074-D1082.
34. *Elanco Compudose™ (estradiol implant)*. Available from: https://www.elancolabels.com/us/compudose.

35. Lamb, G.C. and T. Maddock, *Feed Efficiency in Cows.* Florida Beef Cattle Short Course, 2009: p. 35-42.
36. *SYNOVEX® H (testosterone propionate and estradiol benzoate)*. Available from: https://www.zoetisus.com/_locale-assets/mcm-portal-assets/msds_pi/pi/synovex_h.pdf.
37. *SYNOVEX® S (progesterone and estradiol benzoate implants)*. Available from: https://www2.zoetisus.com/content/_assets/docs/vmips/package-inserts/synovex_s.pdf.
38. Lowinson, J.H., *Lowinson and Ruiz's Substance Abuse: A Comprehensive Textbook*. 2011: Lippincott Williams & Wilkins.
39. *Revalor ®-200 (trenbolone acetate and estradiol)*. Available from: https://www.merck-animal-health-usa.com/channel-content/species/cattle/revalor-200.
40. *RALGRO® Implants for Beef Cattle*. Available from: https://www.merck-animal-health-usa.com/species/cattle/products/ralgro-cattle.
41. *Elanco HeifermaX™ 500*. Available from: https://www.elancolabels.com/us/heifermax-500.
42. Debeljak, Ž., et al., *Analytical bias of automated immunoassays for six serum steroid hormones assessed by LC-MS/MS.* Biochemia medica, 2020. **30**(3): p. 030701.
43. Alder, L., et al., *Residue analysis of 500 high priority pesticides: Better by GC–MS or LC–MS/MS?* Mass Spectrometry Reviews, 2006. **25**(6): p. 838-865.
44. Costain, R.M., et al., *Identification of hormone esters in injection site in muscle tissues by LC/MS/MS.* Food Additives & Contaminants: Part A, 2008. **25**(12): p. 1520-1529.
45. Zhou, H., et al., *Simultaneous measurement of total estradiol and testosterone in human serum by isotope dilution liquid chromatography tandem mass spectrometry.* Analytical and Bioanalytical Chemistry, 2017. **409**(25): p. 5943-5954.
46. McLachlan, J.A., *Environmental signaling: from environmental estrogens to endocrine-disrupting chemicals and beyond.* Andrology, 2016. **4**(4): p. 684-694.
47. Cima, G. *Residues rare in meat, FSIS official says.* October 5, 2019 [cited 2020 August 5]; Available from: https://www.avma.org/javma-news/2019-10-15/residues-rare-meat-fsis-official-says.
48. Johnson, R. *The U.S.-EU Beef Hormone Dispute.* 2012 [cited 2020 November 30].
49. Debruyckere, G., C.H. Van Peteghem, and R. De Sagher, *Influence of the consumption of meat contaminated with anabolic steroids on doping tests.* Analytica Chimica Acta, 1993. **275**(1-2): p. 49-56.
50. Walpurgis, K., et al., *Dietary Supplement and Food Contaminations and Their Implications for Doping Controls.* Foods, 2020. **9**(8): p. 1012.
51. *Association of Official Racing Chemists Guidelines for the Minimum Criteria for Identification by Chromatography and Mass Spectrometry*. 2016.
52. Courant, F., et al., *Exposure assessment of prepubertal children to steroid endocrine disruptors. 2. Determination of steroid hormones in milk, egg, and meat samples.* Journal of Agricultural and Food Chemistry, 2008. **56**(9): p. 3176-3184.
53. Hsieh, M.-K., et al., *Electrochemical Detection of Zeranol and Zearalenone Metabolic Analogs in Meats and Grains by Screen-Plated Carbon-Plated Disposable Electrodes.* Food and Nutrition Sciences, 2013. **4**(07): p. 31-38.
54. Kleinova, M., et al., *Metabolic Profiles of the Mycotoxin Zearalenone and of the Growth Promoter Zeranol in Urine, Liver, and Muscle of Heifers.* Journal of Agricultural and Food Chemistry, 2002. **50**(17): p. 4769-4776.

55. *Improved Sensitivity Through Enhanced Ion Transmission Using an S-Lens on the LTQ Velos Linear Ion Trap*. Thermo Scientific.
56. Fiers, T., et al., *Development of a highly sensitive method for the quantification of estrone and estradiol in serum by liquid chromatography tandem mass spectrometry without derivatization.* Journal of Chromatography B, 2012. **893-894**: p. 57-62.
57. Pesek, J.J. and M.T. Matyska, *Ammonium fluoride as a mobile phase additive in aqueous normal phase chromatography.* Journal of Chromatography A, 2015. **1401**: p. 69-74.
58. Häkkinen, M.R., et al., *Simultaneous analysis by LC–MS/MS of 22 ketosteroids with hydroxylamine derivatization and underivatized estradiol from human plasma, serum and prostate tissue.* Journal of Pharmaceutical and Biomedical Analysis, 2019. **164**: p. 642-652.
59. Gray, B.P., P. Teale, and C.M. Pearce, *Analysis of methyloxime derivatives of intact esters of testosterone and boldenone in equine plasma using ultra high performance liquid chromatography tandem mass spectrometry.* Drug Testing and Analysis, 2011. **3**(4): p. 206-213.
60. Devi, J.L., et al., *Determination of testosterone esters in the hair of male greyhound dogs using liquid chromatography–high resolution mass spectrometry.* Drug Testing and Analysis, 2018. **10**(3): p. 460-473.
61. Furusawa, N., *A Harmless Method for Determining Trenbolone Acetate Together with 17β-Trenbolone in Beef.* Journal of Chromatographic Science, 2009. **47**(3): p. 243-246.
62. Ghosh, C., *Relative matrix effects: A step forward using standard line slopes and ANOVA analysis.* Arabian Journal of Chemistry, 2019. **12**(7): p. 1378-1386.