Equine Plasma Screening for SARMs using Liquid-Liquid Extraction and Triple Quadrupole LC-MS

BY

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THESIS

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This thesis is dedicated to my family, as their unconditional love and endless support made all things possible.

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SARMs	Selective androgen receptor modulators
LC-MS QQQ	Triple quadrupole liquid chromatography-mass spectrometry
UIC AFTL	University of Illinois at Chicago Analytical Forensic Testing Laboratory
LOD	Limit of detection
WADA	World Anti-Doping Agency
IFHA	International Federation of Horseracing Authorities
LC-MS/MS	Liquid chromatography-(tandem) mass spectrometry
DHT	Dihydrotestosterone
DEA	Drug Enforcement Administration
FDA	Food and Drug Administration
LC-MS	Liquid chromatography-mass spectrometry
GC-MS	Gas chromatography-mass spectrometry
UHPLC-QToF-MS	Ultra-high performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry
LC-HRMS	Liquid chromatography high resolution mass spectrometry
HPLC	High performance liquid chromatography
EIC	Extracted ion chromatogram
QC	Quality control

SUMMARY

In the world of sports doping, testosterone and other androgenic steroids have been popular with those trying to gain a competitive edge. However, as these compounds can have negative side effects and increase the risk of prostate cancer in humans, there has been a search for alternative compounds that will still provide the positive aspects of steroids without the drawbacks. Selective androgen receptor modulators, (SARMs), have been shown to increase muscle mass and bone density without significant effects on the prostate. Combined with their easy accessibility due to the internet, this has slated SARMs to be the next big class of drugs to be abused in sports doping.

As it stands, few cases of SARMs in equine doping samples have been reported, but that number can be expected to grow as more SARMs continue to be developed. Without properly validated methods in place, doping control labs cannot properly detect and report on these compounds, and abuses could go unnoticed and undisciplined. Currently, laboratories that test doping control samples are moving towards the use of triple quadrupole liquid chromatography-mass spectrometry (LC-MS QQQ), as it can screen for multiple classes of compounds and has increased sensitivity over past methodologies.

The study performed in this work combined a liquid-liquid extraction method with LC-MS QQQ in for the detection of three SARM compounds, Andarine, Ostarine, and LGD-4033, in equine serum samples. The method was validated according to '*Validation Requirements for Methods Using Instrumental Analysis*' Standard Operating Procedure #AFTL GE 005-04 from the University of Illinois at Chicago Analytical Forensic Testing Laboratory (UIC AFTL). Each sample was spiked with known concentrations of the drugs of interest. The sample then received internal standard solution and methyl tert-butyl ether before being mixed by rotorack. Samples

were then centrifuged, and the top layer was transferred to a new tube. The top layers were dried down, reconstituted, and transferred to a well plate for analysis on LC-MS QQQ.

Results showed that the extraction was successful in recovering the drugs of interest from the plasma samples. There was a suppression of signal for LGD-4033 and Ostarine due to matrix effects, but the Andarine signal was enhanced. Extraction recovery was high for Andarine, Ostarine, and LGD-4033, calculated to be 95%, 81%, and 75%, respectively. The limits of detection (LOD) for Andarine and Ostarine were below 0.0019 ng/mL, and LOD was 0.0039 ng/mL for LGD-4033. Quantitative requirements as set in the method validation procedure were met for all three drugs. Total precision for all three drugs at high and low concentrations was less than 15%. At higher concentrations, the total precision was improved, ranging from 6.60-10.83%. Bias for all three drugs at high and low concentrations never came above the procedural cutoff of 25%, staying below 6% for all data sets.

Based on the results obtained, a liquid-liquid extraction procedure followed by analysis using LC-MS QQQ is a recommended screening protocol for Andarine, Ostarine, and LGD-4033 in equine plasma samples. Implementation of the protocol as outlined in this work would help to detect and prevent the use of SARMs in the horseracing industry.

I. INTRODUCTION

A. Background

Testosterone belongs to a class of steroids known as the androgenic steroids. Androgenic steroidal hormones bind to the transcription factor androgen receptor, which is responsible for gene expression (Haendler and Cleve, 2012). Testosterone and the other androgenic steroidal compounds have been explored extensively in research. However, there are not many approved uses for testosterone and other androgenic steroids due to their undesirable effects on the prostate, cardiovascular system, and serum lipids. The androgen receptor, which is stimulated by these androgenic steroidal hormones, is known to play a major role in prostate cancer, and is suspected to play a role in breast cancer (Haendler and Cleve, 2012). In addition, the administration of testosterone and other androgenic steroids has proved difficult. Multiple methods have been used, each resulting in issues. Subjects given transdermal patches developed rashes. Intravenous administration resulted in fluctuating serum testosterone levels, and little pharmacologic activity was observed with oral administration due to quick metabolism by the liver (Chen et al., 2005).

Selective androgen receptor modulators were first discovered by Dalton in 1998 (Grata et al., 2011). SARMs act in a method similar to the androgenic steroid hormones, but they are selective about which tissues they stimulate (Haendler and Cleve, 2012). As mentioned previously, androgen receptors are known to play a key role in prostate cancer. SARMs, as the name implies, are selective in the androgen receptors that they act on in the body. Compared to testosterone, SARMs have less stimulation at androgen receptors in androgenic tissues such as the prostate, but equal or greater stimulation at androgen receptors in anabolic tissues, such as bone and muscle (Haendler and Cleve, 2012). This makes them desirable over nonselective

steroidal androgens, as they carry less risk of prostate-related complications, but can have the same, if not greater, anabolic effect.

B. Statement of the Problem

As SARMs are well suited to building muscle mass, they are an ideal candidate for illicit use with athletes and those in the horse racing industry. The presence of SARMs in human doping samples has been documented since 2011 (Thevis et al., 2011; Grata et al., 2011), and the first case of a SARM in an equine sample was reported by Cawley in 2015 (Cawley et al., 2016). As the World Anti-Doping Agency (WADA) has banned the use of SARMs in human competition (WADA, 2018), and the International Federation of Horseracing Authorities (IFHA) has banned the use of SARMs in horse racing (IFHA, 2019), the detection of SARMs in doping control samples is necessary to prevent their use in competition. Several SARMs have been characterized in human doping control samples (Thevis and Schänzer, 2018), but SARMs in equine subjects have yet to gain extensive research. While recent studies have examined the metabolism of various SARMs in horses (Hansson et al., 2015; Hansson et al., 2016), analytical methodology to target and confirm the presence of multiple SARMs in equine plasma has yet to be developed.

C. Significance of the Problem

With the knowledge that SARMs pose a significant risk to the integrity of the horse racing industry, forensic laboratories specializing in equine toxicology must have validated methods in place for the detection of such compounds. Prior to this work, UIC AFTL did not have a validated method in place for the detection and confirmation of SARMs in equine plasma samples. Past research of SARMs in equine plasma has utilized liquid chromatography-(tandem) mass spectrometry (LC-MS/MS), but a fully validated method has yet to emerge.

D. **Purpose of the Study**

The purpose of this study is to validate an accurate, reliable, and reproducible method for the detection and confirmation of three SARMs, Andarine, Ostarine, and LGD-4033, in equine plasma using a liquid-liquid extraction method and liquid chromatography-triple quadruple mass spectrometry. The method was validated using the guidelines in the UIC AFTL Standard Operating Procedure '*Validation Requirements for Methods Using Instrumental Analysis*' #AFTL GE005-04.

E. **Significance of the Study**

With validated methods in place to detect SARMs in equine plasma samples, laboratories can be prepared to identify and report findings of SARMs with confidence. Having these procedures in place will hopefully deter the use of these drugs in the horse racing industry.

II. CONCEPTUAL FRAMEWORK AND RELATED LITERATURE

A. Conceptual Framework

1. Testosterone

Androgens, primarily testosterone, are the driving force behind male development and male sexual characteristics (Chen et al., 2005). Testosterone, seen in Figure 1 below (National Center for Biotechnology Information d), is necessary for development during puberty, as well as sexual differentiation and maintenance of the prostate, testis, and epididymus. It is also responsible for the secondary sexual characteristics seen in males, such as facial hair, a lowered voice, and increased muscle mass (Haendler and Cleve, 2012). Testosterone has been used as treatment in male hormone replacement therapy, hypogonadism, and delayed puberty (Chen et al., 2005).

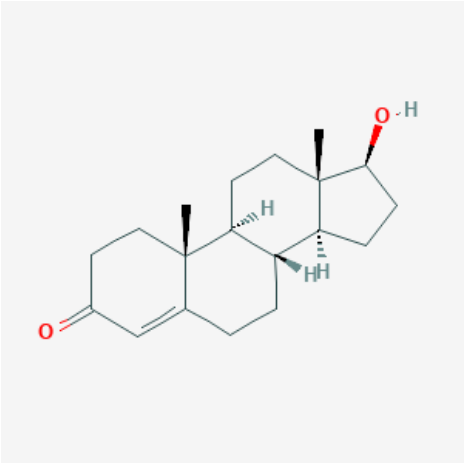


Figure 1. Chemical structure for testosterone.

Approximately 6-8% of testosterone is converted to dihydrotestosterone (DHT) by 5α-reductase, with very high concentrations occurring in the prostate (Chen et al., 2005; Aikawa et al., 2015). DHT is the most potent of the naturally occurring androgens, as it shows the most androgen receptor agonist activity (Aikawa et al., 2015). With a high concentration of DHT being formed and binding to androgen receptors in the prostate, the risk of prostate cancer is increased. In addition, 0.3% of testosterone is converted to estradiol by aromatase enzymes (Chen et al., 2005), which may also contribute to abnormal prostate growth (Haendler and Cleve, 2012).

2. **How SARMs Differ**

5α-reductase does not act on SARMs (Chen et al., 2005), therefore preventing the formation of the amplified androgen product DHT in areas such as the prostate (Grata et al., 2011). Therefore, there is reduced activity at the androgen receptors in areas such as the prostate, leading to a lesser potential for prostate cancer. In addition, unlike testosterone, SARMs cannot undergo aromatization to estradiol, further reducing the risk of abnormal prostate growth (Haendler and Cleve, 2012). Therefore, SARMs possess several qualities that make them desirable replacements for testosterone and androgenic steroids.

3. **Selectivity of SARMs**

Theories about the selectivity of SARMs have suggested that their specificity is due to conformational differences that they induce in the androgenic receptors or that their structures activate different signaling cascades within the cells (Bhasin, 2015; Chen et al., 2005). In muscle tissues or bone, SARMs recruit may co-activators that lead to activation of transcriptional factors for genes responsible for anabolism. This leads to increased muscle mass and greater bone strength, the main desirable property of SARMs. However, in other tissues,

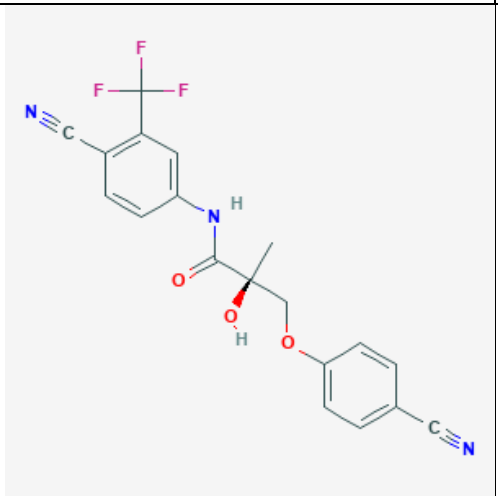
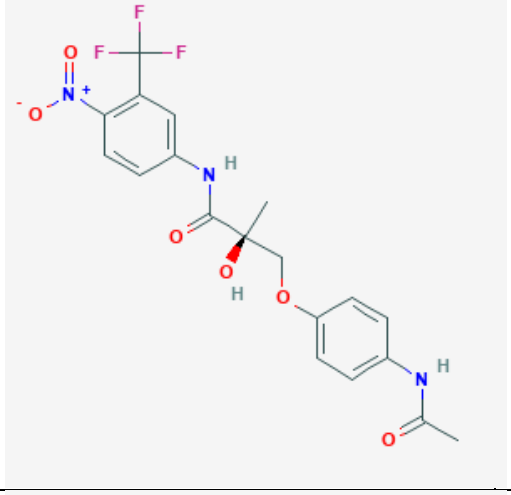
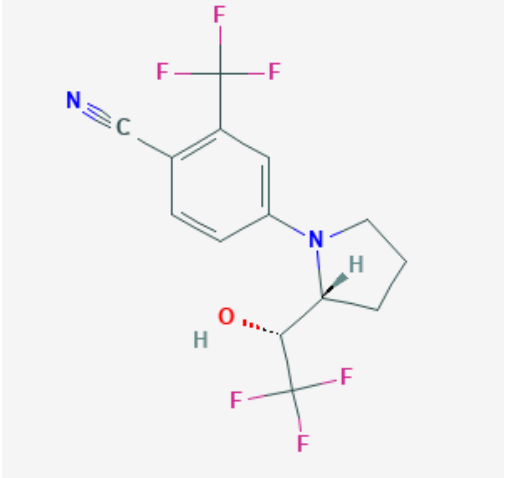
SARMs may recruit a co-suppressor that prevents transcription of anabolic genes (Choi and Lee, 2015). In areas such as the prostate, this is desirable, as increased prostate size may contribute to prostate cancer (Miner et al., 2007). In addition, researchers have isolated three key components that make a SARM favorable to binding to an androgen receptor with high affinity: hydrogen bonding between the SARM and a specific receptor side chain, a structure that contains similarities to the 3-keto group of testosterone, and hydrophobic interactions between the SARM and receptor (Gao et al., 2005a). Some SARMs have been shown to inhibit follicle stimulating hormone and luteinizing hormone, suggesting potential use as male contraceptives (Bhasin, 2015), as their suppression of two of the hormones involved in hypothalamus-pituitary-testis axis prevents spermatogenesis. Due to their binding to androgen receptors in bone, they have also been explored as agents in the prevention and treatment of osteoporosis, to help strengthen bones and increase bone mineral density (Chen et al., 2005). SARMS have also shown to inhibit the proliferation of androgen receptor and estrogen receptor positive breast cancer by suppressing signaling of the estrogen receptors (Ziyang et al., 2017).

4. Classes of SARMs

Multiple classes of SARMs exist that all exhibit similar anabolic properties. Aryl propionamides were originally outlined in 1999 (Zhang and Sui, 2013), and many successful SARMs, such as Andarine (Gao and Dalton, 2007) and Ostarine (Zhang and Sui, 2013) belong to this class. Another class of successful SARMs is the quinolines class, with the majority being developed by Ligand Pharmaceuticals. Most SARMs beginning with LGD, such as LGD-3303 are quinolines (Zhang and Sui, 2013). An exception to this is LGD-4033, which is a classified as a pyrrolidinyl-benzonitrile (Thevis and Schänzer, 2014). The structures and classes of the compounds used in this study can be seen in Table 1 (National Center for Biotechnology

Information c; National Center for Biotechnology Information a; National Center for Biotechnology Information b).

TABLE I: STRUCTURE AND CLASS OF SARMS IN THIS STUDY

Compound	Structure	Class
Ostarine		Aryl propionamide
Andarine		Aryl propionamide
LGD-4033		Pyrrolidinyl-benzonitrile

5. Rise of SARMs

SARMs' ability to increase muscle has led to their rise in popularity both in the research field and the illicit drug trade. In research, SARMs have been shown to help prevent muscle wasting, as they bind to androgenic receptors in muscles and lead to anabolic activity. The effect of this administration is a gain in muscle mass and muscle strength (Chen et al., 2005). Due to this property, SARMs, like the androgenic steroids they are based on, are popular with athletes (Grata et al., 2011). Like their predecessors, SARMs lead to anabolic activity, which athletes desire for their ability to build lean muscle and improve muscle function (Aikawa et al., 2015). However, they do not possess the same risks, such as DHT conversion in the prostate, associated with traditional steroids and testosterone use (Dalton et al., 2011). This makes them popular for abuse in those wanting to gain significant lean muscle, such as body builders. However, use of SARMs gives athletes an unfair advantage over their competitors, and WADA banned the use of all SARMs in sports in 2008. Currently, SARMs are only available on internet black-market sites, as there are no approved pharmaceutical SARM products (Hansson et al., 2015).

6. Illicit Use

Currently SARMs exist in a legal gray area: they are not explicitly deemed illegal by the federal government, but this could soon change. As of April 2018, a bill was entered into the 115th Congress to have the Drug Enforcement Administration (DEA) schedule SARMs, including Andarine, Ostarine, and LGD-4033, as schedule III substances. This is the same schedule where anabolic steroids are currently classified, and if the bill is passed, it would make the possession of SARMs a felony offense. In addition, the Food and Drug Administration (FDA) would be required to notify the DEA if a dietary supplement was found to contain a

SARM (Sen. Hatch, Orrin G. [R-UT], 2018). Despite being banned by WADA (WADA, 2018), and their possible future as a schedule III substance, SARMs have begun to appear in athlete drug screening assays. In 2010, Grata reported the first case of SARM abuse during competition testing. Andarine and one of its metabolites, referred to as M5, were detected in the urine sample of a female athlete. However, no Andarine was found in the blood sample drawn the day prior, leading the agency to conclude the athlete had administered the drug after the blood draw, but prior to the urine sample (Grata et al., 2011). Another case was reported in a German laboratory in 2013, where a minor league baseball player's urine tested positive for Andarine and the M5 metabolite (Starcevic et al., 2013). The SARM LGD-4033 has been documented in six human doping samples in the United States and Canada, leading Cox and Eichner to examine LGD-4033 and its metabolites in four human urine samples (Cox and Eichner, 2017). A male subject in a 2011 study admitted to self-administration of approximately 1 mg of Ostarine, and his sample was used to study Ostarine and its metabolites in human urine (Thevis et al., 2011).

7. **The Horseracing Industry**

The popularity of SARMs extends past humans into the horseracing industry, where they are administered to racehorses to build lean muscle mass. Jockeys and owners most likely obtain SARMS from online retailers similar to the ones mentioned previously. The drugs are administered to the horses with the expectation that they will have the same desirable effects of increased muscle growth and muscle strength as seen in humans. However, while the administration of SARMs in humans has been documented since their creation (Grata et al., 2011), administration in horses has yet to gain a significant amount of research. Human metabolism of SARMs has been studied, but this data are not completely applicable to equine test subjects, as metabolic patterns are likely to differ between the two species (Hansson et al.,

2015). An example of such a difference was seen in past experiments with SARMs, as Andarine was shown to have different bioavailability in rats and dogs. This was based on differences in the major metabolic pathway, which was noted as an amide hydrolysis in rats and B-ring deacetylation in dogs (Mohler et al., 2009). *In vitro* systems have been explored as potential options to avoid animal testing, but a study of Ostarine in calf liver microsomes showed that livers cannot provide a good qualitative profile of the metabolites that would be produced *in vivo* (Hansson et al., 2015).

8. **International Federation of Horseracing Authorities**

The possible emergence of SARMs is unsurprising, given the long list of substances that have been previously abused in the horseracing industry. These substances have included anti-diabetics, anti-ulcer medications, COX-3 inhibitors, sedatives, corticosteroids, anabolic steroids, diuretics, and more. In order to combat the large volume of current and emerging substances, the governing body of the horse racing industry, IFHA, defined what substances are banned. Article 6 of the International Agreement on Breeding, Racing, and Wagering published by IFHA bans any substance that has an effect on a mammal's body. This law effectively covers every substance that could be administered to a horse, whether it was created at the time of legislation or later. However, the definition is so broad that it includes medications possibly necessary for medical purposes (Wong et al., 2011). Due to this flaw, authorities are allowed to show discretion in circumstances in which medications are administered (Scarth et al., 2011). Without any accepted medical use of SARMs in horses, the article effectively bans all SARMs from the industry, which is why testing procedures are necessary to detect them. Even if SARMs are banned in the industry under the legislation, if there are no validated sample testing protocols in place that will detect SARMs, then trainers and

owners could feel free to administer them, knowing that they will not be caught. This is why properly validated, rigorously tested methods are in place to prevent this abuse from happening.

9. **Extraction Methods**

At the current time, SARMs have been extracted from multiple bodily fluids and byproducts using various methods in many different animals. Cesbron used solid phase extraction to examine Ostarine and its metabolites in the urine and feces of calves (Cesbron et al., 2016). Cox and Eichner used a liquid-liquid extraction on urine samples from human subjects taking the SARM LGD-4033 (Cox and Eichner, 2017), while Thevis used a solid phase extraction on human urine samples containing Ostarine (Thevis et al., 2011). For equine plasma samples, both solid phase extraction (Cawley et al., 2016) and protein precipitation methods have been used (Hansson et al., 2016; Hansson et al., 2018).

10. **Instrumental Methods**

Liquid chromatography-mass spectrometry (LC-MS) was originally developed as a complimentary technique to gas chromatography-mass spectrometry (GC-MS). While GC-MS is still widely used today, LC-MS has risen in popularity in clinical and forensic toxicology laboratories (Remane et al., 2016). Within the last 20 years, LC-MS has shown the capacity to target a large range of compounds in both urine and plasma equine samples (Wong et al., 2011). Many studies examining SARMs in urine and plasma samples have made use LC-MS/MS (Hansson et al., 2015; Hansson et al., 2016; Thevis et al., 2010a; Thevis et al., 2010b; Thevis et al., 2011), however, there is a lack of studies using LC-MS QQQ for the detection of SARMs in biological matrices.

B. Related Literature

1. Andarine

One of the most successful and earliest SARMs developed was Andarine, also known as S-4 (Girgis, 2015). Andarine, developed in 2002, showed high binding affinity to the androgen receptor when compared to other SARMs in development (Yin et al., 2003). Clinical data from Andarine indicated androgen receptor stimulation of 93%, one of the highest percentages among the SARMs developed (Yin et al., 2003). During its development, the prostates, seminal vesicles, and levator ani muscles were reduced in a group of castrated rats. These muscles were chosen as the prostate and seminal vesicles would monitor the growth due to the androgenic effects of the drug, while the levator ani muscle would monitor the anabolic effects of the drug. Administration of various dosage levels ranging from 0.1 mg/day to 1.0 mg/day of S-4 to the castrated rats caused slight increases in prostate and seminal vesicle size, but dramatic increases in the levator ani muscle. When administered 0.75 mg/day, the mass of the levator ani muscle grew from 40.9% to 101% when compared to the non-castrated control rats. Not only did Andarine administration recover the lost muscle, it also was able to increase it even further to surpass the control rats. The researchers concluded that S-4 not only had androgenic properties, but it had powerful anabolic properties, as it was able to maintain the muscle weight of the levator ani muscle in castrated rats with low doses of approximately 0.3 mg/day. The final data produced from the study were encouraging for the research team. Andarine had produced less potency and efficacy in androgenic tissues when compared to testosterone, but more potency and nearly the same efficacy for its anabolic effects (Yin et al., 2003). Additional studies showed that in administration to rats, Andarine had quick and

complete oral absorption with low doses, as well as a half-life estimated to be anywhere from 2.6 hours to 5.3 hours (Narayanan et al., 2008).

Since the development of SARMs, there has been a search for an "ideal" SARM: an orally active compound taken once a day, with anabolic effects on muscle and bone, and no effects on the prostate (Mohler et al., 2009). For a while, Andarine was thought to meet these requirements. Firstly, Andarine has shown that it is orally active. It showed 100% bioavailability in rats orally administered a 10 mg/kg dose, as blood plasma levels reflected little to no loss of the active drug upon reaching the blood (Kearbey et al., 2004). There was only a slight decrease to 91% in dogs, which researchers explained as metabolic differences in the species. Secondly, it has been shown to only require once daily doses. The clearance rate of Andarine and volume of distribution data showed that Andarine was not degraded or excreted quickly, and it was deposited to peripheral tissues and not deposited in fat (Mohler et al., 2009). Finally, the anabolic effects of Andarine have been proven throughout several studies. A study of Andarine in rats showed decreased bone loss in the vertebrae, an increase in the cortical bone thickness, and an improvement in bone strength (Kearbey et al., 2007). The anabolic effects of Andarine on muscle were proven during its development and subsequent studies. Additional studies after development confirmed that Andarine was able to restore muscles in castrated rats to equal or greater mass than control non-castrated rats. By studying the soleus muscles of sacrificed rats Andarine was also shown to improve muscle strength in rats. Further research was able to show that in rats, Andarine can lead to traditionally desirable weight loss by its ability to decrease body fat mass while increasing lean body mass (Mohler et al., 2009). As for the prostate, Andarine has been shown to have a lesser effect than testosterone (Yin et al., 2003). Despite Andarine presenting the "perfect" profile as the ideal SARM, it was not chosen for advanced

clinical development. After phase I studies, Andarine was passed over in favor of Ostarine for advanced development due to side effects only described as "visual disturbances" during the course of the trials (Mohler et al., 2009; Starcevic et al., 2013).

2. **Ostarine**

Of all the SARMs studied to date, Ostarine, otherwise known as GTx-024, MK-2866, Enobosarm, and S-22 (Zhang and Sui, 2013), has seen the most research and has made it the farthest of any SARM in clinical trials. It is currently in phase III of clinical trials for muscle wasting (Thevis and Volmer, 2018). In a study of elderly men and postmenopausal women, Ostarine was shown to increase lean muscle and improve physical muscle function with no adverse androgenic effects. The researchers in the study posited that Ostarine would be an ideal treatment for muscle wasting for patients with chronic diseases (Dalton et al., 2011). In addition, subjects in the study had reduced glucose levels, insulin levels, and insulin resistance, suggesting a potential use in patients either at risk for or with diabetes (Narayanan et al., 2008). Ostarine was further studied in cancer patients who exhibited muscle wasting. Patients with cancer cachexia were administered Ostarine for a 16 week period, and researchers saw significant increases in patient lean body mass (Bhasin, 2015). During this Phase II trial, patients were better able to climb stairs and reported better quality of life (Handlon et al., 2016). Patients in this trial also saw decreases in cholesterol levels, placing them in a lower risk category for cardiovascular issues (Narayanan et al., 2008). An additional group of patients with small cell lung cancer saw similar results with administration of Ostarine (Bhasin, 2015).

3. **LGD-4033**

Ligand Pharmaceuticals, Inc. has developed a wide range of SARMs, all preceded with LG or LGD. LGD2226 was one of their first SARMs tested. In preclinical trials, LGD2226

was shown to increase bone mineral density and improve bone strength and structure, but was discontinued shortly after its development for undisclosed reasons. A follow up to LGD2226 was LGD2941, which showed increased bioavailability when compared to its predecessor. Ligand Pharmaceuticals, Inc. then went on to manufacture LGD-3303 and LGD-4033. In clinical trials with rats, LGD-3303 improved bone mineral density and femur bone strength (Narayanan et al., 2008). LGD-4033 exhibited anabolic effects on bone and muscle and partial agonist effects on the prostate in rat trials. In female rats, dosages of 0.3 mg/kg body weight were shown to produce increased skeletal muscle mass. In addition, LGD-4033 indicated inherent tissue selectivity, as the selectivity of the drug was shown to be independent of local drug concentration (Zhang and Sui, 2013). When tested in healthy, young male adults, LGD-4033 was shown to increase bone strength, bone mineral density, and bone formation after a 21 day period (Bhasin, 2015). In addition, subjects administered the drug showed increases in their lean body mass and in leg press strength and a loss of body fat when compared to the placebo group. These increases were most significant in the group administered 1.0 mg/day (Basaria et al., 2013). Based on this success, the company moved LGD-4033 onto trials for the treatment of cancer patients with muscle wasting (Handlon et al., 2016).

4. **Other SARMs**

Other companies producing SARMs have seen various successes with bone and muscle strength. Bristol-Myers Squibb & Co. Inc produced BMS-564929, which started clinical trials for age-related functional decline. However, the compound was shown to have issues with selectivity, as it produced irregular dose-response curves and prostate sizing (Narayanan et al., 2008). In women over 65, the SARM MK-0773, developed by Merck Pharmaceuticals (Zhang and Sui, 2013), was shown to improve lean body mass, but subjects showed no signs of leg

strength improvement or other physical functions. However, the women in the study did have decreased levels of HDL (Bhasin, 2015). GSK2420A, from GlaxoSmithKline, was shown to return muscle function and strength in castrated rats after 28 days of treatment (Narayanan et al., 2008).

5. **Black Market**

The possible side effects of unapproved SARMs has not stopped athletes from using these drugs to try and gain lean muscle mass. In 2014, the German Bureau of Customs Investigation seized 297.6 kilograms and 3,800 ampules of various doping agents. This included 1,000 grams of Ostarine, and one unit of LGD-4033 purchased from an internet retailer (Krug et al., 2014). 30 mL of Andarine can be purchased from online retailers in the form of an oily liquid for approximately $100. However, online sites are careful to list that the product is not intended for human use, as it has not been officially launched or passed clinical trials. The illicit nature of these drugs is evident upon delivery. In an instance where researchers ordered two units of Andarine, one of the packages that arrived at the lab was labeled as containing moisturizer and green tea extract. Neither the box nor bottle contained any information regarding its contents (Thevis et al., 2009), highlighting the risks that are present with the distribution of illicit, unapproved pharmaceutical substances. When purchasing illicit products online, there is no quality control or protocols in place to ensure product safety. The source of the substance is virtually unknown, and there is no guarantee that the substance received is the product ordered or if it is safe. In a study by Van Wagoner, forty-four SARM products were purchased from twenty-one internet retailers for chemical analysis. Of these forty-four, only eighteen contained the compound and dosage that appeared on the label. The rest of the products contained additional undisclosed SARMs, different concentrations, a different compound, or no detectable active

compound at all (Van Wagoner et al., 2017). Researchers in a different study purchases LGD-4033 from an online retailer at a time when the chemical structure had not yet been released by the pharmaceutical company (Thevis et al., 2015). A separate group obtained LGD-4033 from an internet retailer and confirmed the presence of LGD-4033 without the presence of other potentially harmful contaminants (Geldof et al., 2017). A similar study by a group of German researchers confirmed the presence of LGD-4033 in a black market product in 2014 (Krug et al., 2014).

6. **Testosterone Metabolism**

The metabolism of testosterone is similar across species. 5α-reductase is present in humans (Chen et al., 2005) as well as horses (Corbin et al., 2016). This enzyme leads to the formation of DHT, an active metabolite of testosterone (Aikawa et al., 2015). Inactive metabolites of testosterone have been shown to vary between species. An *in vitro* study using liver microsomes from humans, horses, and dogs, showed formation of metabolites androstenedione, 6β-hydroxytestosterone, and 11β-hydroxytestosterone in all three species. Humans had higher concentrations of 6β-hydroxytestosterone, while horses and dogs had higher concentrations of androstenedione and 11β-hydroxytestosterone. In addition, while the concentration of androstenedione remained at the same concentration for humans and dogs, androstenedione concentration was shown to increase with time in horses (Zielinski and Mevissen, 2015).

7. **Andarine Metabolism**

The metabolites formed by Andarine are based on its structure and how that structure is broken down by hepatic enzymes in the liver and other processes involving cystolic and microsomal enzymes. The metabolism of Andarine occurs in two phases. The first phase

metabolism occurs in the liver, and the second phase is glucuronidation and sulfonation of these metabolites (Kuuranne et al., 2008). Ten metabolites of Andarine have been observed during both *in vitro* and *in vivo* studies. These metabolites were first identified during *in vitro* studies using microsomal and human liver preparations by Kuuranne in 2008 (Kuuranne et al., 2008). In 2010, a urine sample was obtained from a male subject who admitted to self-administration of 75 mg of internet purchased Andarine per week (Thevis et al., 2010b). Of the ten metabolites observed, the metabolite M5 stood out. M5 was identified as being an aglycon of the M4 glucuronide metabolite. In addition to being one of the most prominent metabolites observed, M5 could be observed using gas chromatography-mass spectrometry (GC-MS) after derivatization. Two other metabolites, the deactetylated products M2 and M3, were detectable but not well suited to gas chromatography. Therefore, the researchers focused on detection of Andarine and the M5 metabolite (Thevis et al., 2010b).

This difference in the metabolism of SARMs, particularly Andarine, is evident when comparing human administration and metabolism studies with the current studies done in equine subjects. The major metabolites seen in horses are hydrolysis and sulfonation products as opposed to glucuronidation as seen in humans (Garg et al., 2018). A study by Hansson from 2014 in horses found that only three of the nine Andarine metabolites seen in their equine subjects were also observed in the human liver microsome studies performed by Kuuranne (Hansson et al., 2015). This number was lowered to one of the nine when comparing to human administration research performed by Thevis in 2010 (Hansson et al., 2015). This one metabolite identified in their Andarine research was named M6b. M6b has an identical structure to the M5 metabolite that was identified as the main metabolite ion of use in humans (Hansson et al., 2015; Thevis et al., 2010b). However, the M6b equine metabolite was not the metabolite with the

greatest detectable signal intensity. The equine metabolites M3 and M4, seen in Figure 2 below, had the highest signal intensities in mass spectrometry (Hansson et al., 2015). M3 was noted as being a hydroxylation and sulfonation of the M1 metabolite, a biotransformation product that seems to be a fragment of the original SARM molecule. M4 was noted as being a dihydroxylation and sulfonation of the M1 metabolite (Hansson et al., 2015). Further research on the metabolism of SARMs showed that the process occurs in two stages corresponding to these structural differences from the parent compound.

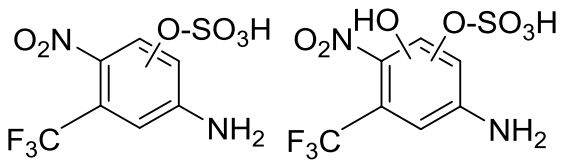


Figure 2. Equine Andarine metabolites M3 (left) and M4 (right).

Aryl-propionamide SARMs first undergo hydrolysis, producing an intermediate molecule. This intermediate then undergoes phase I selective oxidation via P450 enzymes, and then phase II sulfonation or glucuronidation (Thevis et al., 2010a; Garg et al., 2018). In addition, the human metabolism study performed by Thevis showed that Andarine was detected in the urine after 36 hours post administration of the drug (Thevis et al., 2010b). In the original equine study performed by Hansson, the parent compound was only detectable 3 hours after administration (Hansson et al,. 2015). However, Hansson performed another study in 2016,

where the parent compound was detectable in equine plasma for 12 hours after administration. The M3 metabolite of Andarine previously discovered by the group was detectable in equine plasma for 18 hours, and the M4 metabolite was detectable for 12 hours (Hansson et al., 2016). Therefore, while the parent compound may be an appropriate compound to target in cases human testing, it may not be as valuable in equine testing if the sample was taken more than 12 hours after administration of Andarine. After 12 hours, the metabolite M3 is considered to be the best molecule to target when searching for signs of Andarine in plasma.

8. **Ostarine Metabolism**

Thirteen metabolites of Ostarine were identified during a 2010 *in vitro* study using human liver microsomes. Metabolite M3, a O-dephenylation of the parent compound, was identified as good metabolite to target in doping control analysis, as it was a metabolite for the SARM S23 as well as Ostarine. M3 was later confirmed to be a good metabolite to target for detection of Ostarine. When Ostarine was administered to canine test subjects, M3 along with the parent drug Ostarine, were still detectable in urine samples 48-72 hours after administration (Thevis et al., 2010a). When administered to horses, seven metabolites were detected, and only two metabolites, M6 and M9, were identical to those found in humans. However, only the equine metabolite termed M3 seen in Figure 3 below, a hydroxylation and sulfonation of the parent, was deemed to a good target for doping analysis as it was detectable by ultra-high performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (UHPLC-QToF-MS) for 48 hours (Hansson et al., 2015). The same metabolite proved to be a good target in equine plasma as well, as M3 and the parent drug were detectable for up to 18 hours post administration (Hansson et al., 2016).

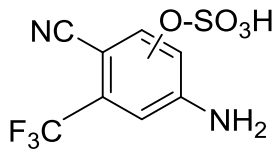


Figure 3. Equine Ostarine metabolite M3.

9. LGD-4033 Metabolism

The metabolites of LGD-4033 were studied using human liver microsomes by Geldof in 2015. A black-market form of LGD-4033 purchased off the internet was tested for the presence of the drug of interest as well as its purity. After using this drug with human liver microsomes for an *in vitro* study, the group detected five metabolites using liquid chromatography high resolution mass spectrometry (LC-HRMS), and only one metabolite via GC-MS. The five metabolites detected using LC-HRMS had all undergone phase I metabolism. Of these five, only one metabolite, identified as M2, underwent phase II metabolism to form a glucuronide conjugate (Geldof et al., 2017). When Cox and Eichner analyzed urine samples from athletes using LGD-4033, they discovered two additional metabolites. They also posited that the metabolite known as M4, a dihydroxylation previously seen in the *in vitro* studies, would be a good metabolite to target for anti-doping laboratories. (Cox and Eichner, 2017). When LGD-4033 was administered to horses by Hansson in 2018, they saw eight total metabolites in the urine, with two metabolites, M4 and M5, being identical to those seen in human urine and *in vitro* liver microsome samples. The parent compound was only detectable in equine urine after undergoing hydrolysis. However, with the hydrolysis step, the parent was detectable for up to 72 hours, longer than any of the metabolites. The metabolism of LGD-4033 was also studied in

plasma samples, and six of the eight urine metabolites were also present in the plasma samples. For plasma samples, the metabolite termed 5a, seen in Figure 4 below, could still be observed up to 5 hours after administration (Hansson et al., 2018).

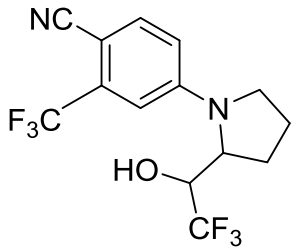


Figure 4. Equine LGD-4033 metabolite M5a.

10. SARM Dosage Levels

The dosage of SARMs also plays an important role in their effects and metabolism profiles. In addition, this is another area where human studies may vary from animal studies. Humans, especially those interested in gaining significant amounts of muscle, are more likely to self-administer dosages well above the therapeutic range noted in literature. During the development stages, rats were administered 0.1-1.0 mg/day of S-4 (Yin et al., 2003). Studies on the effect of Andarine on hormone levels listed the therapeutic dose of Andarine as 0.5 mg/day. For determination of bioavailability, the levels were raised, and the rats were administered doses of 10 mg/kg of body weight, which led to 100% bioavailability (Mohler et al., 2009). During trials examining the effect of Andarine on body composition and muscle strength, rats were

administered dosages of 3 mg/kg body weight or 10 mg/body weight (Gao et al., 2005b). These dosage levels of 3 mg/kg body weight and 10 mg/kg body weight seem to be the most common rate dosages throughout several other trials on the anabolic effects of Andarine (Mohler et al., 2009).

Appropriate human dose levels of SARMs are difficult to determine, as human trials with discontinued SARMs such as Andarine appear to have decreased due to adverse side effects, such as visual disturbances (Starcevic et al., 2013). Even for SARMs that are still in clinical development, those abusing the products have no obligation to follow clinical dosing recommendations. In addition, human users may not know the true amount they are administering due to false labeling as seen in past studies of internet purchased SARM products (Van Wagoner et al., 2017). The little data on SARMs in humans comes from few clinical trials and those who were caught or admitted to taking the drugs. In human trials, healthy adult males were orally administered 0.1, 0.3, or 10 mg LGD-4033/day for 21 days, and an increase in lean body mass was seen with increasing dosages (Basaria et al., 2013). Research has been performed on urine samples from men who admitted to self-administering SARMs. One man had ingested 75 mg of Andarine on a weekly basis (Thevis et al., 2010b), and another admitted to consuming 11 mg of Ostarine three days prior to providing his sample (Thevis et al., 2011). Studies from athletes caught doping with Andarine failed to disclose the amount that the athlete was taking, possibly because the athletes themselves did not provide the amount that was consumed. Volunteers in a different experiment were administered a single 15 mg dose of Andarine, and their urine samples confirmed the presence of Andarine 12 hours after administration (Dmitrieva et al., 2018).

While SARM doping has been seen in several human cases, there has only been one documented case of a SARM in an equine sample. Cawley examined a routine doping control sample from an equine subject in 2015, finding Andarine in the plasma sample (Cawley et al., 2016). However, researchers are working to be ready when other SARMs, such as S1 and Ostarine, are found in equine subjects (Hansson et al., 2015; Hansson et al., 2016). In these studies, equine dosages were extrapolated from human clinical data with the same or similar SARMs. In 2014, Hansson administered 41.7 mg S-1, 41.7 mg Andarine, and 33.3 mg Ostarine to nine adult female horses weighing 504-590 kg (Hansson et al., 2015). In 2016, Hansson again administered 41.7 mg S-1, 41.7 mg Andarine, and 33.3 mg Ostarine to nine adult female horses. This time, they noted that the dosage used was calculated based on prior clinical data with SARMs in humans, (Hansson et al., 2016) where doses of 0.1 mg per individual up to 3 mg per individual were used (Dalton et al., 2011). Horses in a later study by Hansson received a single intravenous dose of 0.1 mg LGD-4033/kg of body weight in order to determine metabolites of the drug in urine and plasma (Hansson et al., 2018).

III. METHODS AND MATERIALS

A. Scope

The purpose of method validation is to ensure that the method is appropriate for the specified compounds and instrumentation and to prove the accuracy, reliability, and reproducibility of the given method.

B. Reagents and Materials

Negative control serum was purchased from BioIVT (Westbury, NY). High performance liquid chromatography (HPLC) grade methyl tert-butyl ether, water, acetonitrile, and formic acid were purchased from Fisher Scientific (Waltham, MA). Analytical standards for Andarine, Ostarine (MK-2866), and LGD-4033 were purchased from Caymen Chemical (Ann Arbor, MI). The analytes were made into individual standard solutions at concentrations of 1 mg/mL, 100 ng/mL, and 1 ng/mL. Butalbital-d5 was purchased from Sigma-Aldrich (St. Louis, MO) and used as an internal standard for Andarine, Ostarine, and LGD-4033. Butalbital-d5 was made into an internal standard solution in water with a concentration of 500 ng/mL. The internal standard was chosen due to its negative ionization, which was necessary with the analysis method used.

A sample of Andarine powder was also purchased from online retailer SARMsPharm for future studies in administration. When quantified, the purity was found to be close to 90%, which is less than traditional laboratory grade standards.

C. Sample Preparation

Samples were prepared according to the '*Serum Screening Extraction*' protocol provided by UIC AFTL. Half a milliliter of equine serum, spiked with the analytes of interest was allocated to screw-top glass test tubes. One hundred microliters of internal standard in water was added to each test tube. Three milliliters of methyl tert-butyl ether were added to each test tube

and capped tightly. The tubes were mixed by rotorack for 10 minutes, followed by centrifuging at 3000 rpm for 10 minutes. After centrifuging, the top organic layer of methyl tert-butyl ether was transferred to a glass test tube using a disposable transfer pipet. The samples were then evaporated to dryness in a 40°C water bath under a stream of nitrogen.

Samples were reconstituted using one hundred microliters of 95:5 0.2% formic acid in water: acetonitrile, transferred to a well plate with insert, and placed in the autosampler tray for LC-MS QQQ injection.

D. **Instrumentation**

All analyses were conducted using an Agilent 1200 Series HPLC coupled to an Agilent 6400 Series Mass Spectrometry Triple Quadrupole in negative ionization mode. The column used was an Agilent Poroshelll 120 EC-C_{18} column 2.1 mm x 100 mm, 2.7 µM pore size. Column temperature was set to 40°C and mobile phase flow was set to 500 µL/min.

E. **LC Parameters**

Solvents used were (A) 0.2% formic acid in water and (B) 10% water in acetonitrile. For negative ionization, initial conditions of 45% B were held for 0.5 minutes. A gradient was employed between 0.5 and 15 minutes from 45% B to 100 % B and held at 100 % for 1 minute.

F. **MS-QQQ Parameters**

Drying gas temperature was set to 350°C. Gas flow was set to 12 L/min, and the nebulizer was set to 50 psi. Electrospray ionization was used for all compounds. Data was collected in Dynamic Multiple Reaction Monitoring Mode (DMRM). Data for precursor and product ions were also collected for each compound. Data analysis was performed with Agilent Masshunter Quantitative software. Full LC-MS QQQ method parameters can be seen in the Appendix.

G. Procedure

The method was validated according to the UIC AFTL Standard Operating Procedure #AFTL GE005-04 '*Validation Requirements for Methods Using Instrumental Analysis*'.

1. Validation Requirements for Qualitative Methods

a. Specificity

Three blank serum samples were run with three serum samples spiked with Andarine, Ostarine, and LGD-4033 at a concentration of 50 ng/mL. After analysis, specificity was determined if each compound was not identified in the blank serum samples and was positively identified alone and in the presence of other compounds

b. Ion Suppression and Enhancement

Three sets of samples were prepared, each containing 2 µg/mL Andarine, Ostarine, and LGD-4033. The first set (Set A) was neat standards. The second and third sets of samples were blank serum samples spiked before (Set C) and after (Set B) the extraction was performed. Each set contained three replicates. Peak areas for each compound were used to calculate matrix effect, extraction recovery, and process efficiency using the following equations:

$$\text{Matrix Effect (\%)} = (\text{Set B}/\text{Set A}) \times 100 - 100 \quad (1)$$

$$\text{Extraction Recovery (\%)} = (\text{Set C}/\text{Set B}) \times 100 \quad (2)$$

$$\text{Process Efficiency (\%)} = (\text{Set C}/\text{Set A}) \times 100 \quad (3)$$

c. Limit of Detection

Blank serum samples were spiked with decreasing concentrations of Andarine, Ostarine, and LGD-4033 by 1:2 serial dilutions at 1 ng/mL, 0.5 ng/mL, 0.25 ng/mL, 0.125 ng/mL, 0.0625 ng/mL, 0.03125 ng/mL, 0.0156 ng/mL, 0.0078 ng/mL, 0.0039 ng/mL, and

0.0019 ng/mL, for a total ten samples. Samples were extracted and analyzed. The limit of detection was determined when the signal to noise ratio of any of the ions used for identification fell below 5:1.

d. **Carryover and Contamination**

Three blank serum samples were spiked with Andarine, Ostarine, and LGD-4033 at concentrations higher than normally expected in an unknown sample (50 ng/mL). Spiked samples were extracted together with three blank serum samples and analyzed. Three blank serum samples were injected directly after each spiked sample and analyzed for compounds of interest.

e. **Stability**

i. **Freeze Thaw**

Two sets of blank serum samples were spiked with Andarine, Ostarine, and LGD-4033 at 5 ng/mL in triplicate. One set was stored at -20°C for 48 hours, removed, thawed, and placed back in the freezer three times. The other set of samples was stored in the refrigerator for the entire duration of the freeze-thawing cycles. After the third thaw, both sets of samples were extracted and analyzed, and the peak areas of the two sets were compared.

ii. **Long Term**

Two sets of blank serum samples were spiked with Andarine, Ostarine, and LGD-4033 at 5 ng/mL in triplicate. One set was stored at -20°C for 60 days. The other set of samples was stored in the refrigerator for the entire time. After 60 days, the frozen samples were thawed. Both sets of samples were extracted and analyzed, and the peak areas of the two sets were compared.

iii. **Bench Top**

Two sets of blank serum samples were spiked with Andarine, Ostarine, and LGD-4033 at 5 ng/mL in triplicate. One set was stored at room temperature for 24 hours. The other set of samples was stored in the refrigerator for the entire time. Both sets of samples were extracted and analyzed, and the peak areas of the two sets were compared.

iv. **Processed Samples**

Two sets of blank serum samples were spiked with Andarine, Ostarine, and LGD-4033 at 5 ng/mL in triplicate. Both sets were extracted, and one set was immediately analyzed. The second set was stored on the instrument (as though prepared for injection) for 48 hours and then analyzed, and the peak areas of the two sets were compared.

2. **Validation Requirements for Quantitative Methods**

a. **Lower Limit of Quantitation**

Blank serum samples were spiked with Andarine, Ostarine, and MK-2866. The limit of quantitation was determined when the signal-to-noise of any of the ions of the compounds of interest used for quantitation fell below 10:1.

b. **Calibration Model (Linearity)**

The range of the calibration curve depends on the purpose of the method but shall cover the majority of concentrations to be expected in unknown samples. The curve shall contain at least 6 points. The curve was established by analyzing spiked samples at decreasing concentrations (50 ng/mL, 25 ng/mL, 12.5 ng/mL, 6.25 ng/mL, 3.125 ng/mL, 1.5625 ng/mL, 0.781 ng/mL, and 0.39 ng/mL) and plotting the resulting responses versus the corresponding concentrations. The curve was generated using simple linear regression and evaluated by residual plots (r^2 values). The r^2 value shall be 0.95 or higher and be based on

curves run at least three different times. The curves in this study contained eight points and were run five different times.

c. **Precision**

Blank serum samples were spiked with Andarine, Ostarine, and LGD-4033 at two different levels: low (1 ng/mL) and high (20 ng/mL). These concentrations must fall within the lowest (0.39 ng/mL) and highest (50 ng/mL) concentrations of the calibration curve. There were 6 replicates per concentration, and the procedure was repeated 5 separate days. The quantitative results were analyzed to create one-way ANOVA tables for each compound at both concentration levels. Those numbers were then used to calculate the following precision values and expressed in percentage:

i. **Intra-day Precision**

$$RSD_r(\%) = \frac{\sqrt{MS_{wg}}}{\bar{x}} \times 100 \quad (4)$$

Where RSD_r = intra-day precision (expressed as a percentage), MS_{wg} = the mean square within groups (determined by one-way ANOVA), and $\bar{x}$ = the grand mean.

ii. **Inter-day Precision**

$$RSD_{bg} = \frac{\sqrt{\frac{MS_{bg} - MS_{wg}}{n}}}{\bar{X}} \times 100 \quad (5)$$

Where RSD_{bg} = inter-day precision (expressed as a percentage), MS_{bg} = the mean square between groups (determined by one-way ANOVA), MS_{wg} = the mean square between groups (determined by one-way ANOVA), n = the number of observations in each group, and $\bar{X}$ = the grand mean.

iii. **Total Precision**

$$RSD_{I(F)}(\%) = \frac{\sqrt{\frac{MS_{bg} + (n+1)MS_{wg}}{n}}}{\bar{X}} \times 100 \quad (6)$$

Where $RSD_{I(F)}$ = total precision, MS_{bg} = the mean square between groups (determined by one-way ANOVA), MS_{wg} = the mean square within groups (determined by one-way ANOVA), n = the number of observations in each group, and $\bar{X}$ = the grand mean. Values for intra-day precision, inter-day precision, and total precision should be less than 20% at each concentration.

d. **Accuracy**

The same controls used for precision were used for accuracy. The quantitative results were used to calculate the total accuracy of the method at each concentration. The results are shown as a percentage, and the calculation was as follows:

$$Bias = \frac{\bar{X}-X}{X} \times 100 \quad (7)$$

Where $\bar{X}$ = the grand mean and X = the theoretical value. Bias at each concentration should be less than 25%.

e. **Uncertainty**

Uncertainty was calculated using the same data used to calculate precision and accuracy. The Simplified *Guide to the Expression of Uncertainty in Measurement* (GUM) approach was used.

IV. RESULTS

A. Qualitative Requirements

Qualitative results were obtained for specificity, ion suppression/enhancement, LOD, carryover and contamination, and stability. Extracted ion chromatograms (EIC) for Andarine and the internal standard butalbital-d5 can be seen in Figure 5 below.

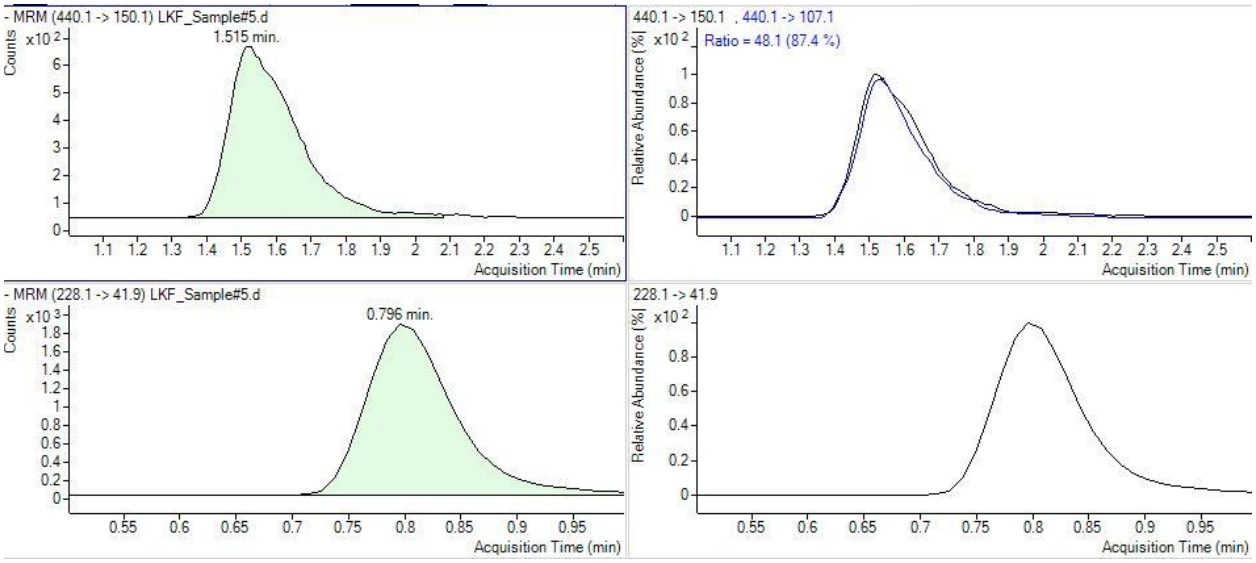


Figure 5. EIC for Andarine (top) and internal standard butalbital-d5 (bottom).

Retention times for the compounds were 1.5 and 0.8, respectively. For Andarine, selective monitoring of precursor ions was at a mass of 440.1 (parent ion) and product ions at mass 107.1. Extracted ion chromatograms for Ostarine and the internal standard butalbital-d5 can be seen in Figure 6 below.

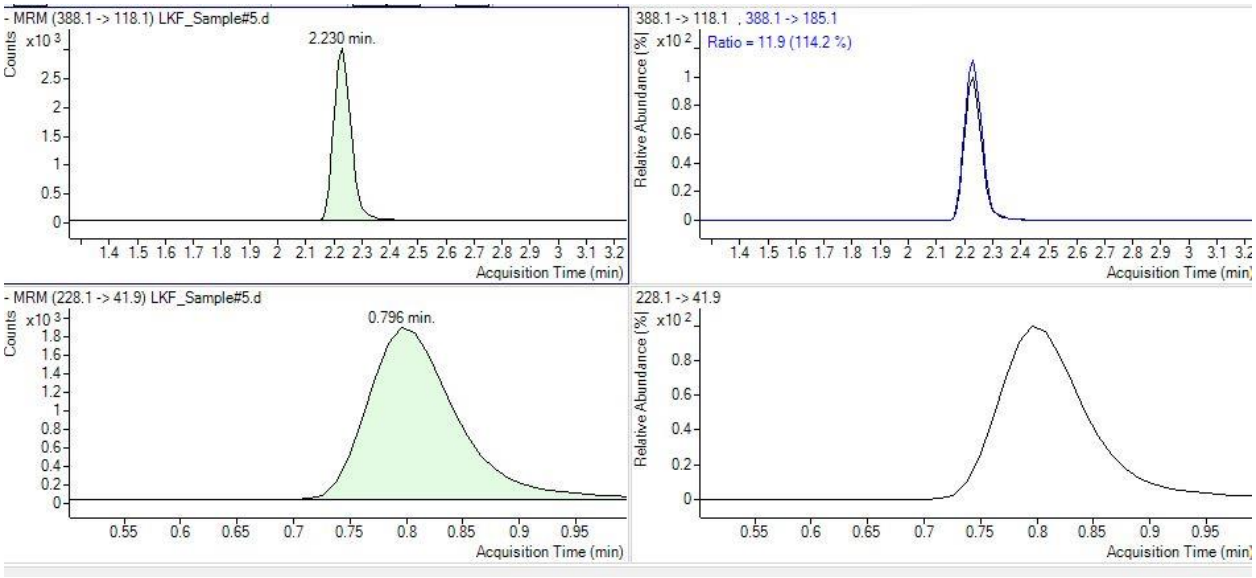


Figure 6. EIC for Ostarine (top) and internal standard butalbital-d5 (bottom).

Retention times for the compounds were 2.2 and 0.8, respectively. For Ostarine, selective monitoring of precursor ions was at a mass of 388.1 (parent ion) and product ions at mass 185.1. Extracted ion chromatograms for LGD-4033 and the internal standard butalbital-d5 can be seen in Figure 7 below.

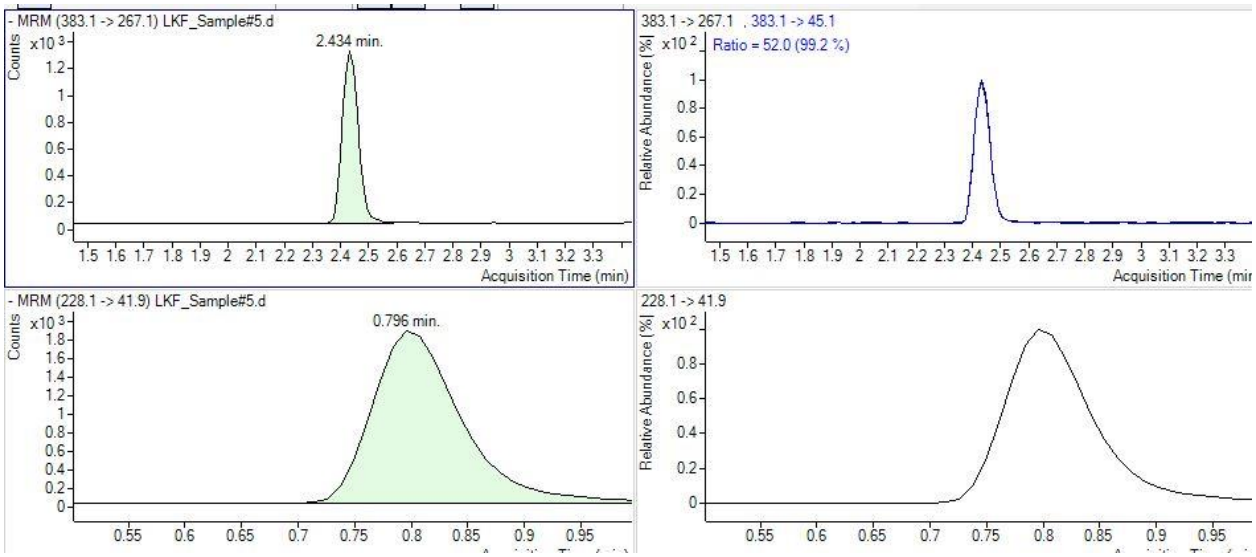


Figure 7. EIC for LGD-4033 (top) and internal standard butalbital-d5 (bottom).

Retention times for the compounds were 2.4 and 0.8, respectively. For LGD-4033, selective monitoring of precursor ions was at a mass of 383.1 (formate adduct) and product ions at mass 45.1. The peaks for the Ostarine and LGD-4033 as seen in Figures 6 and 7 exhibited normal distribution and minimal tailing. The peak for Andarine, as seen in Figure 5, exhibited tailing, but was determined to not interfere with the drug's detection. To determine specificity, each drug was identified alone and samples with all three compounds, and none of the drugs were found in blank samples.

The raw data used to calculate the matrix effect and extraction efficiencies can be viewed in Table II. The matrix effect and extraction efficiencies calculated for each compound are seen in Table III. Matrix suppression was seen with Ostarine and LGD-4033, with values of –29% and –37% (Equation 1) respectively. Matrix enhancement of 52% was seen with Andarine. Extraction recovery ranged from 75% for LGD-4033 to 95% (Equation 2) for Andarine. The

overall process efficiency was 50% for LGD-4033, 57% for Ostarine, and 144% (Equation 3) for Andarine, indicating that the liquid-liquid extraction process enhanced response for Andarine, but suppressed the response for Ostarine and LGD-4033.

As LOD is determined when the signal to noise ratio falls below 5:1, the LOD was set at 0.0039 ng/mL for LGD-4033, and 0.0019 for Andarine and Ostarine. Table IV shows the decreasing concentrations and signal to noise ratios obtained for each concentration. As the signal to noise ratio never fell below 5:1 for Andarine and Ostarine, the true LOD may be lower than what was observed in this study.

TABLE II: RAW DATA USED FOR MATRIX EFFECT AND EXTRACTION EFFICIENCIES

Raw Data			Area Counts (Peak Areas)								
			Pre-Extraction (C)			Post Extraction (B)			Standard (A)		
Extraction Efficiencies	Precursor (m/z)	Transition (m/z)	Replicate 1	Replicate 2	Replicate 3	Replicate 1	Replicate 2	Replicate 3	Replicate 1	Replicate 2	Replicate 3
Andarine	440	107	17695	17728	20003	16234	21098	21099	13163	11202	14066
Ostarine	388	185	302157	266159	281474	333348	366823	344190	492334	581723	404443
LGD-4033	383	45	178124	188049	228885	271923	272049	248941	390702	452057	351455

TABLE III: MATRIX EFFECT AND EXTRACTION EFFICIENCIES

Calculations			
Extraction Efficiencies	**Matrix Effects (%)**	**Extraction Recovery (%)**	**Process Efficiency (%)**
Andarine	52.04	94.86	144.22
Ostarine	-29.36	81.37	57.48
LGD-4033	-33.60	75.05	49.83

TABLE IV: LIMIT OF DETECTION

Sample (ng/mL)	Andarine Results			Ostarine Results			LGD-4033 Results		
Name	RT	Resp.	S/N	RT	Resp.	S/N	RT	Resp.	S/N
Blank	2.21	1036.95	24.81	2.17	1.26	0.45	3.05	2.54	0.51
-C	2.20	440.24	7.36	2.16	7.08	0.13	2.61	54.16	0.88
Standard	2.21	241047.53	3965.95	2.47	2210809.44	774.26	2.61	1497252.27	364.05
1	2.21	48089.84	496.33	2.47	614961.68	1962.42	2.61	255136.27	524.86
0.5	2.21	11390.87	5128.65	2.47	347574.04	1133.57	2.61	114897.54	278.25
0.25	2.21	3337.66	882.63	2.47	107135.68	1234.52	2.61	47291.31	179.63
0.125	2.21	2444.46	263.71	2.47	44004.07	661.12	2.61	23458.94	70.33
0.0625	2.21	797.09	193.23	2.47	29812.62	10443.22	2.61	11278.81	63.11
0.03125	2.21	927.54	349.28	2.47	16150.38	718.02	2.61	5603.70	60.76
0.0156	2.21	1195.12	11.80	2.46	6663.30	190.55	2.61	3737.70	19.66
0.0078	2.22	642.80	13.39	2.47	3697.63	907.04	2.61	1891.36	23.12
0.0039	2.20	730.84	12.56	2.47	2004.37	440.82	2.61	840.89	8.55
0.0019	2.21	493.48	135.09	2.47	1270.45	382.10	2.47	16.60	0.17

Carryover and contamination were established by injecting and analyzing 3 blank samples immediately after a high concentration spiked sample. This process was repeated three times. As the signal to noise for the compounds detected in all three blank samples was below 5:1, the risk of carryover and contamination was deemed to be extremely low, and can be ignored.

Stability was determined by having spiked samples undergo several different processes, and the average peak responses of the samples were compared (Tables V, VI, VII). Andarine was the most stable of the three compounds, showing little change in response when undergoing freeze-thaw cycles or being left on the instrument. It showed enhanced response when left in long term frozen storage or on the bench-top at room temperature. Ostarine also remained stable throughout the freeze-thaw, had enhanced response in long term frozen storage, but performed poorly when left on the bench-top at room temperature or on the instrument. LGD-4033 showed little change when undergoing freeze-thaw and bench-top storage, showed greater response when put in long term frozen storage, but had very poor response when left on the instrument. Overall, the results indicate that samples can be left in frozen storage for up to 60 days if they cannot be analyzed immediately.

Qualitative requirements for butalbital-d5 were previously performed by UIC AFTL, and the results can be seen in Table VII.

TABLE V: STABILITY OF ANDARINE

	Area Counts		
	Control	**Freeze Thaw**	**Average Response [a]**
Replicate 1	26060.83	26712.60	1.18
Replicate 2	16179.67	23471.11	
Replicate 3	20545.75	24027.18	
	Area Counts		
	Control	**Long Term**	**Average Response [a]**
Replicate 1	10536.67	17822.61	1.30
Replicate 2	12056.28	14362.20	
Replicate 3	11245.07	11906.92	
	Area Counts		
	Control	**Bench Top**	**Average Response [a]**
Replicate 1	47605.71	37966.22	1.60
Replicate 2	49198.18	101559.11	
Replicate 3	18124.49	44367.05	
	Area Counts		
	Control	**Processed**	**Average Response [a]**
Replicate 1	26060.83	29720.01	1.10
Replicate 2	16179.67	17355.45	
Replicate 3	20545.75	21704.89	

[a] Average response was calculated by dividing the average area count for the processed samples by the average area count for the control samples.

TABLE VI: STABILITY OF OSTARINE

	Area Counts		
	Control	**Freeze Thaw**	**Average Response [a]**
Replicate 1	152513.39	220957.95	1.21
Replicate 2	146027.44	157218.67	
Replicate 3	149406.50	164075.51	
	Area Counts		
	Control	**Long Term**	**Average Response [a]**
Replicate 1	154663.61	284390.94	1.66
Replicate 2	178551.72	311741.49	
Replicate 3	172722.56	244035.63	
	Area Counts		
	Control	**Bench Top**	**Average Response [a]**
Replicate 1	204830.36	83579.36	0.64
Replicate 2	263093.85	221500.53	
Replicate 3	362353.09	222199.24	
	Area Counts		
	Control	**Processed**	**Average Response [a]**
Replicate 1	152513.39	91013.61	0.43
Replicate 2	146027.44	52338.36	
Replicate 3	149406.50	51090.41	

[a] Average response was calculated by dividing the average area count for the processed samples by the average area count for the control samples.

TABLE VII: STABILITY OF LGD-4033

	Area Counts		
	Control	**Freeze Thaw**	**Average Response [a]**
Replicate 1	130056.01	153571.30	1.06
Replicate 2	137513.36	117863.13	
Replicate 3	117097.59	136150.45	
	Area Counts		
	Control	**Long Term**	**Average Response [a]**
Replicate 1	56345.68	78659.41	1.35
Replicate 2	65539.85	94623.82	
Replicate 3	64559.29	78090.61	
	Area Counts		
	Control	**Bench Top**	**Average Response [a]**
Replicate 1	162411.01	5587.56	1.07
Replicate 2	121735.13	134062.80	
Replicate 3	6906.02	171518.58	
	Area Counts		
	Control	**Processed**	**Average Response [a]**
Replicate 1	130056.01	27871.93	0.17
Replicate 2	137513.36	19460.55	
Replicate 3	117097.59	17279.95	

[a] Average response was calculated by dividing the average area count for the processed samples by the average area count for the control samples.

TABLE VIII: QUALITATIVE RESULTS OF INTERNAL STANDARD

Butalbital-d5 [a]	**Result**
Extraction Efficiency	75%
Matrix Effects	-90.80%
Bench Top	106%
Processed	75%
Freeze Thaw	90%
Long Term	85%
LOD	6 ng/mL

[a] Qualitative requirements for butalbital-d5 were previously performed by UIC AFTL

B. **Quantitative Results**

Calibration curves for Andarine, Ostarine, and LGD-4033 were created by 1:2 serial dilutions ranging from 50 ng/mL to 0.39 ng/mL. The samples were analyzed and linear calibration curves were obtained by linear regression. Figures 8, 9, and 10 show the linear calibration curves generated for Andarine, Ostarine, and LGD-4033, respectively. Eight calibration points were used for each curve, and 5 replicates of the curve were generated. All R^2 values obtained were 0.95 or higher, and many were above 0.98. The curves were weighted to 1/x, giving more weight to the bottom points of the calibration curve because more calibrators fell in the lower half.

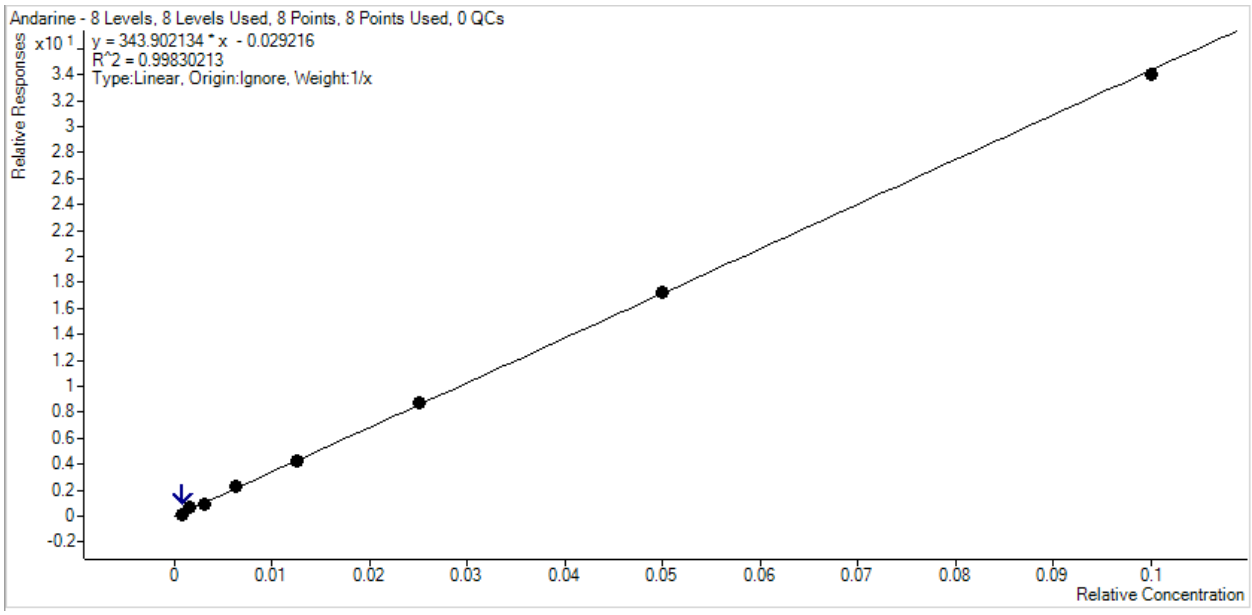


Figure 8. Calibration curve for Andarine. Relative concentration units are ng/mL and relative responses units are area counts.

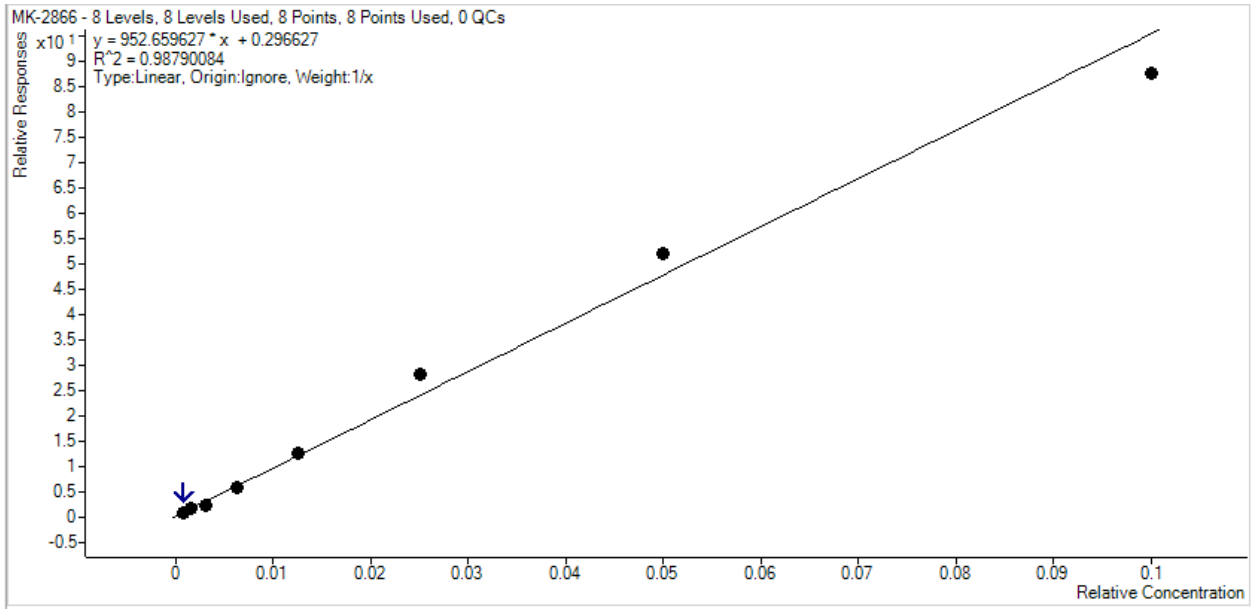


Figure 9. Calibration curve for Ostarine. Relative concentration units are ng/mL and relative responses units are area counts.

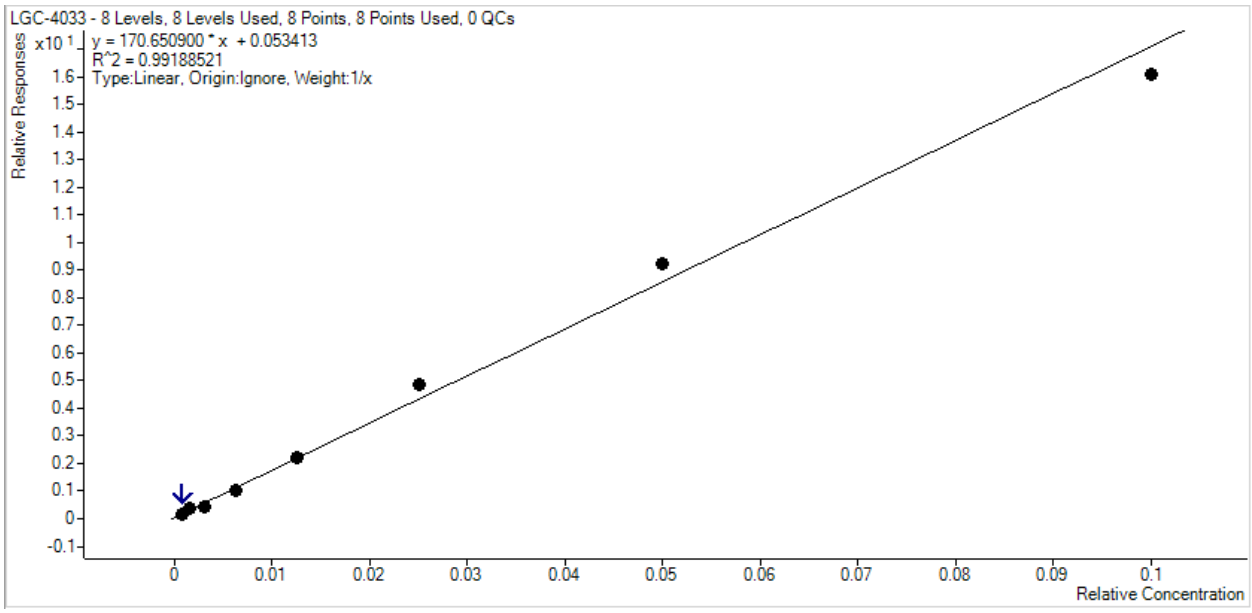


Figure 10. Calibration curve for LGD-4033. Relative concentration units are ng/mL and relative responses units are area counts.

Quality control (QC) samples at high (20 ng/mL) and low (1 ng/mL) levels within the calibration curve were run in addition to the calibrators. Raw data from the five separate QC run that was used to calculate accuracy, precision, and uncertainty is seen in Table IX. Sum, mean, and range of each group are included with the raw data. Values three standard deviations above the mean or three standard deviations below the mean, highlighted in yellow, were deemed as outliers and were excluded from further calculations. The adjusted range for each group of data, excluding these outliers, is also seen in Table IX.

Table X contains the statistics for the high and low QCs. These statistics were generated using the data from Table IX, excluding the outliers as previously mentioned. The average standard uncertainty for the Andarine, LGD-4033, and Ostarine were 11.62%, 11.81%, and 11.08%, respectively. Data from Table IX were also used to create one-way ANOVA tables for

each compound at high and low concentrations. The one-way ANOVA tables for Andarine, LGD-4033, and Ostarine at high (20 ng/mL) concentrations are seen in Tables XI, XII, and XIII, respectively. The one-way ANOVA tables for Andarine, LGD-4033, and Ostarine at low (1 ng/mL) concentrations are seen in Tables XIV, XV, and XVI, respectively. Intra-day precision (Equation 4), inter-day precision (Equation 5), and total precision (Equation 6), seen under each ANOVA table, were calculated for each drug at high and low concentrations. These values, expressed as percentages, were all below the 20% threshold. In fact, all of the precision values were less than 15%. Precision statistics at the low QC concentration were slightly higher than their high QC counterparts, indicating better method precision at the higher concentration.

Accuracy (Equation 8), expressed as a percentage, was calculated for Andarine, LGD-4033, and Ostarine at high and low concentrations using the QC data from Table X. The accuracy value for each drug at high or low concentrations are seen under the corresponding ANOVA table. Accuracy values for each drug at high and low concentrations were below the 25% threshold. Bias for Andarine and LGD-4033 for the high concentration fell below 1%, and was just above 5% for Ostarine. All three compounds exhibited a bias of 5% at the low concentration.

Uncertainty for each compound as calculated using the Uncertainty Budget Form (Table XVII). The same QC sample data used for accuracy and precision calculations was used in the determination of uncertainty. The QC data for each drug was the largest source of uncertainty, as many of the other sources of uncertainty were ignored, as they were negligible. The QC repeatability values as seen in Table XVII were obtained by averaging the total precisions of each drug from their high and low concentrations. The expanded uncertainties for Andarine, LGD-4033, and Ostarine were 10.25%, 12.00%, and 11.28% respectively.

TABLE IX: RAW DATA FOR PRECISION AND ACCURACY CALCULATIONS

QC High Data (20 ng/mL)	**8/26/19**			**9/3/19**			**9/5/19**			**9/6/19**			**9/9/19**		
	Andarine	LGD-4033	Ostarine	Andarine	LGD-4033	Ostarine	Andarine	LGD-4033	Ostarine	Andarine	LGD-4033	Ostarine	Andarine	LGD-4033	Ostarine
	17.98	20.13	17.69	17.64	17.51	18.26	20.39	20.64	21.62	20.82	18.98	22.67	20.87	19.16	20.60
	18.78	23.45	18.99	18.77	19.90	21.43	23.93	23.95	26.48	19.48	16.35	19.20	20.53	19.90	21.31
	20.15	27.07	22.15	19.52	20.23	21.97	20.95	17.78	20.77	21.88	19.96	24.00	20.11	19.41	20.46
	15.42	17.83	14.44	21.86	22.02	24.22	19.57	17.89	18.93	19.79	17.34	20.50	20.72	19.15	20.26
	16.98	22.51	18.26	20.49	20.42	23.09	28.05	27.35	27.89	29.17	28.14	32.58	21.83	22.67	22.22
	10.38	13.11	10.59	18.98	19.39	20.73	19.31	17.55	19.50	22.53	20.67	25.59	22.78	23.32	24.30
Sum	99.69	124.10	102.12	117.27	119.48	129.70	132.19	125.16	135.20	133.67	121.43	144.55	126.82	123.62	129.15
Mean	16.62	20.68	17.02	19.54	19.91	21.62	22.03	20.86	22.53	22.28	20.24	24.09	21.14	20.60	21.52
Range	9.77	13.95	11.55	4.22	4.51	5.96	8.73	9.81	8.96	9.68	11.79	13.38	2.67	4.18	4.04
Adjust Range[a]	3.17	5.63	4.45				4.61	6.40	7.55	3.04	4.31	6.40			
QC Low Data (1 ng/mL)	**8/26/19**			**9/3/19**			**9/5/19**			**9/6/19**			**9/9/19**		
	Andarine	LGD-4033	Ostarine	Andarine	LGD-4033	Ostarine	Andarine	LGD-4033	Ostarine	Andarine	LGD-4033	Ostarine	Andarine	LGD-4033	Ostarine
	4.04	3.55	2.91	0.90	0.94	1.01	0.83	0.85	0.81	0.96	1.27	1.01	1.08	1.04	1.01
	1.08	0.88	1.01	0.98	1.01	1.07	0.67	0.84	0.86	0.83	1.13	1.01	1.07	1.14	1.13
	1.20	1.05	1.02	0.96	0.90	1.02	0.81	1.07	1.02	0.77	0.95	0.81	1.04	0.94	0.84
	1.04	0.58	1.02	0.76	0.76	0.70	0.80	0.90	0.89	1.23	1.49	1.42	1.03	0.80	1.04
	1.18	0.96	1.02	0.85	0.98	0.94	1.07	1.00	0.96	0.76	0.91	0.74	0.98	0.93	1.11
	1.09	0.79	0.89	0.81	0.93	0.90	0.57	0.71	0.72	0.80	1.10	1.03	0.89	0.87	0.87
Sum	9.63	7.80	7.88	5.27	5.52	5.64	4.76	5.37	5.27	5.35	6.85	6.01	6.09	5.72	5.99
Mean	1.61	1.30	1.31	0.88	0.92	0.94	0.79	0.90	0.88	0.89	1.14	1.00	1.01	0.95	1.00
Range	3.00	2.97	2.02	0.22	0.25	0.37	0.49	0.36	0.31	0.48	0.59	0.68	0.19	0.33	0.29
Adjust Range[a]	0.16	0.47	0.14				0.40				0.36	0.29			

[a] Adjusted range was calculated by ignoring the outliers (highlighted in yellow) in each data set.

TABLE X: HIGH AND LOW QC STATISTICS

QC High Data 20 ng/mL[a]								
	Andarine	LGD-4033	Ostarine			Andarine	LGD-4033	Ostarine
Subgroup Size	6.00	6.00	6.00		Grand Mean	20.11	19.93	21.15
Grand Average	20.11	19.93	21.15		Grand StDev	1.47	2.07	2.08
Average Range	3.54	5.01	5.68		Std Unc.	0.07	0.10	0.10
rxa2	1.71	2.42	2.74					
D4	2.00	2.00	2.00					
UCLX	21.82	22.35	23.89		UCLR	7.10	10.03	11.39
CLX	20.11	19.93	21.15		CLR	3.54	5.01	5.68
LCLX	18.40	17.51	18.41		LCLR	0.00	0.00	0.00

QC Low Data 1 ng/mL[a]								
	Andarine	LGD-4033	Ostarine			Andarine	LGD-4033	Ostarine
Subgroup Size	6.00	6.00	6.00		Grand Mean	0.95	0.95	0.95
Grand Average	0.95	0.95	0.95		Grand StDev	0.15	0.13	0.12
Average Range	0.29	0.35	0.28		Std Unc.	0.16	0.13	0.12
rxa2	0.14	0.17	0.13					
D4	2.00	2.00	2.00					
UCLX	1.09	1.12	1.08		UCLR	0.58	0.71	0.56
CLX	0.95	0.95	0.95		CLR	0.29	0.35	0.28
LCLX	0.81	0.78	0.81		LCLR	0.00	0.00	0.00

Average Std Uncertainty (%)[b]	Andarine	LGD-4033	Ostarine
	11.62	11.81	11.08

[a] Statistics for high and low QC were calculated after the removal of outliers from each data set. UCLX and LCLX (outside 3 standard deviations from the mean) were used to determine outliers.

[b] Average standard uncertainty was determined by averaging the standard uncertainties of each drug at both high and low QC levels.

TABLE XI: ANOVA TABLE FOR ANDARINE AT 20 NG/ML

Anova: Single Factor - Andarine 20 ng/mL						
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
Column 1	4	73.89	18.47	1.79		
Column 2	6	117.27	19.54	2.17		
Column 3	4	80.22	20.05	0.57		
Column 4	5	104.51	20.90	1.72		
Column 5	6	126.82	21.14	0.97		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	22.11	4	5.53	3.73	0.02	2.87
Within Groups	29.61	20	1.48			
Total	51.72	24				
Grand Mean (ng/mL)[a]	20.11					
Intra-day Precision (%)[b]	6.05					
Inter-Day Precision (%)[c]	2.00					
Total Precision (%)[d]	6.60					
Accuracy (%)[e]	0.55					

[a] Grand mean was obtained from Table X for Andarine at 20 ng/mL and was used to determine accuracy

[b] Intra-day precision was calculated using Equation 4 and numbers generated in the one-way ANOVA table

[c] Inter-day precision was calculated using Equation 5 and numbers generated in one-way ANOVA table.

[d] Total precision was calculated using Equation 6 and numbers generated in one-way ANOVA table.

[e] Accuracy was calculated using Equation 7 with the theoretical value at 20 ng/mL.

TABLE XII: ANOVA TABLE FOR LGD-4033 AT 20 NG/ML

Anova: Single Factor - LGD-4033 20 ng/mL						
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
Column 1	4	83.92	20.98	6.37		
Column 2	6	119.48	19.91	2.17		
Column 3	5	97.81	19.56	7.61		
Column 4	5	93.29	18.66	3.22		
Column 5	6	123.62	20.60	3.56		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	15.90	4	3.98	0.92	0.47	2.84
Within Groups	91.08	21	4.34			
Total	106.98	25				
Grand Mean (ng/mL)[a]	19.93					
Intra-day Precision (%)[b]	10.45					
Inter-Day Precision (%)[c]	0.59					
Total Precision (%)[d]	10.83					
Accuracy (%)[e]	0.35					

[a] Grand mean was obtained from Table X for LGD-4033 at 20 ng/mL and was used to determine accuracy

[b] Intra-day precision was calculated using Equation 4 and numbers generated in the one-way ANOVA table

[c] Inter-day precision was calculated using Equation 5 and numbers generated in one-way ANOVA table.

[d] Total precision was calculated using Equation 6 and numbers generated in one-way ANOVA table.

[e] Accuracy was calculated using Equation 7 with the theoretical value at 20 ng/mL.

TABLE XIII: ANOVA TABLE FOR OSTARINE AT 20 NG/ML

Anova: Single Factor - Ostarine 20 ng/mL						
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
Column 1	4	77.09	19.27	3.95		
Column 2	6	129.70	21.62	4.24		
Column 3	4	80.83	20.21	1.48		
Column 4	5	111.97	22.39	6.68		
Column 5	6	129.15	21.52	2.36		
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	27.55	4	6.89	1.81	0.17	2.87
Within Groups	76.03	20	3.80			
Total	103.58	24				
Grand Mean (ng/mL)[a]	21.15					
Intra-day Precision (%)[b]	9.22					
Inter-Day Precision (%)[c]	1.66					
Total Precision (%)[d]	9.72					
Accuracy (%)[e]	5.75					

[a] Grand mean was obtained from Table X for Ostarine at 20 ng/mL and was used to determine accuracy

[b] Intra-day precision was calculated using Equation 4 and numbers generated in the one-way ANOVA table

[c] Inter-day precision was calculated using Equation 5 and numbers generated in one-way ANOVA table.

[d] Total precision was calculated using Equation 6 and numbers generated in one-way ANOVA table.

[e] Accuracy was calculated using Equation 7 with the theoretical value at 20 ng/mL.

TABLE XIV: ANOVA TABLE FOR ANDARINE AT 1 NG/ML

Anova: Single Factor - Andarine 1 ng/mL						
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
Column 1	5	5.59	1.12	0.00		
Column 2	6	5.27	0.88	0.01		
Column 3	5	4.18	0.84	0.02		
Column 4	6	5.35	0.89	0.03		
Column 5	6	6.09	1.01	0.01		
	28					
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.28	4	0.07	4.91	0.01	2.80
Within Groups	0.33	23	0.01			
Total	0.61	27				
Grand Mean (ng/mL)[a]	0.95					
Intra-day Precision (%)[b]	12.63					
Inter-Day Precision (%)[c]	4.72					
Total Precision (%)[d]	13.90					
Accuracy (%)[e]	5.00					

[a] Grand mean was obtained from Table X for Andarine at 1 ng/mL and was used to determine accuracy

[b] Intra-day precision was calculated using Equation 4 and numbers generated in the one-way ANOVA table

[c] Inter-day precision was calculated using Equation 5 and numbers generated in one-way ANOVA table.

[d] Total precision was calculated using Equation 6 and numbers generated in one-way ANOVA table.

[e] Accuracy was calculated using Equation 7 with the theoretical value at 1 ng/mL.

TABLE XV: ANOVA TABLE FOR LGD-4033 AT 1 NG/ML

Anova: Single Factor - LGD-4033 1 ng/mL						
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
Column 1	4	3.68	0.92	0.01		
Column 2	6	5.52	0.92	0.01		
Column 3	6	5.37	0.90	0.02		
Column 4	5	5.35	1.07	0.02		
Column 5	6	5.72	0.95	0.01		
	27					
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.10	4	0.02	1.76	0.17	2.82
Within Groups	0.31	22	0.01			
Total	0.41	26				
Grand Mean (ng/mL)[a]	0.95					
Intra-day Precision (%)[b]	12.52					
Inter-Day Precision (%)[c]	2.11					
Total Precision (%)[d]	13.15					
Accuracy (%)[e]	5.00					

[a] Grand mean was obtained from Table X for LGD-4033 at 1 ng/mL and was used to determine accuracy

[b] Intra-day precision was calculated using Equation 4 and numbers generated in the one-way ANOVA table

[c] Inter-day precision was calculated using Equation 5 and numbers generated in one-way ANOVA table.

[d] Total precision was calculated using Equation 6 and numbers generated in one-way ANOVA table.

[e] Accuracy was calculated using Equation 7 with the theoretical value at 1 ng/mL.

TABLE XVI: ANOVA TABLE FOR OSTARINE AT 1 NG/ML

Anova: Single Factor - Ostarine 1 ng/mL						
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
Column 1	5	4.97	0.99	0.00		
Column 2	6	5.64	0.94	0.02		
Column 3	6	5.27	0.88	0.01		
Column 4	5	4.60	0.92	0.02		
Column 5	6	5.99	1.00	0.01		
	28					
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.06	4	0.01	1.12	0.37	2.80
Within Groups	0.31	23	0.01			
Total	0.37	27				
Grand Mean (ng/mL)[a]	0.95					
Intra-day Precision (%)[b]	12.16					
Inter-Day Precision (%)[c]	0.79					
Total Precision (%)[d]	12.61					
Accuracy (%)[e]	5.00					

[a] Grand mean was obtained from Table X for Ostarine at 1 ng/mL and was used to determine accuracy

[b] Intra-day precision was calculated using Equation 4 and numbers generated in the one-way ANOVA table

[c] Inter-day precision was calculated using Equation 5 and numbers generated in one-way ANOVA table.

[d] Total precision was calculated using Equation 6 and numbers generated in one-way ANOVA table.

[e] Accuracy was calculated using Equation 7 with the theoretical value at 1 ng/mL.

TABLE XVII: UNCERTAINTY BUDGET FORM

UNCERTAINTY BUDGET FORM: QUANTITATION OF SARMS IN EQUINE SERUM						
Method: Quantitation of SARMs in Equine Serum						
Analyst: Lucy Freitag			Date: 9/10/19			
Sources of Uncertainty (Andarine)	Type A or B	Std. Dev or Outside Limits (%)[a]	Distribution Model	Divisor	Standard Uncertainty (%)	Can it be ignored?
Average Repeatability - QC Data	A	10.25	Student's t	1.73	5.92	N
Calibration of Andarine Stock Std	B	0.02	Rectangular	1.73	0.02	Y
10 mL Vol Flask	B	0.10	Rectangular	1.73	0.06	Y
Pipet 25 uL Butalbital-d5 Std	B	0.50	Rectangular	1.73	0.29	Y
Pipet 5 uL H2O for ISTD	B	0.50	Rectangular	1.73	0.29	Y
Pipet 100 uL of Butalbital-d5 Stock Std	B	0.50	Rectangular	1.73	0.29	Y
Pipet 1 uL of Andarine Stock Std	B	0.50	Rectangular	1.73	0.29	Y
Combined Uncertainty (%):	5.92					
99.7% Confidence Level k value:	3.00					
Expanded Uncertainty (%):	10.25					
Sources of Uncertainty (LGD-4033)	Type A or B	Std. Dev or Outside Limits (%)[a]	Distribution Model	Divisor	Standard Uncertainty (%)	Can it be ignored?
Average Repeatability - QC Data	A	11.99	Student's t	1.73	6.93	N
Calibration of LGD-4033 Stock Std	B	0.02	Rectangular	1.73	0.02	Y
10 mL Vol Flask	B	0.10	Rectangular	1.73	0.06	Y
Pipet 25 uL Butalbital-d5 Std	B	0.50	Rectangular	1.73	0.29	Y
Pipet 5 uL H2O for ISTD	B	0.50	Rectangular	1.73	0.29	Y
Pipet 100 uL of Butalbital-d5 Stock Std	B	0.50	Rectangular	1.73	0.29	Y
Pipet 1 uL of LGD-4033 Stock Std	B	0.50	Rectangular	1.73	0.29	Y
Combined Uncertainty (%):	6.93					
99.7% Confidence Level k value:	3.00					
Expanded Uncertainty (%):	12.00					
Sources of Uncertainty (Ostarine)	Type A or B	Std. Dev or Outside Limits (%)[a]	Distribution Model	Divisor	Standard Uncertainty (%)	Can it be ignored?
Average Repeatability - QC Data	A	11.27	Student's t	1.73	6.51	N
Calibration of Ostarine Stock Std	B	0.02	Rectangular	1.73	0.02	Y
10 mL Vol Flask	B	0.10	Rectangular	1.73	0.06	Y
Pipet 25 uL Butalbital-d5 Std	B	0.50	Rectangular	1.73	0.29	Y
Pipet 5 uL H2O for ISTD	B	0.50	Rectangular	1.73	0.29	Y
Pipet 100 uL of Butalbital-d5 Stock Std	B	0.50	Rectangular	1.73	0.29	Y
Pipet 1 uL of Ostarine Stock Std	B	0.50	Rectangular	1.73	0.29	Y
Combined Uncertainty (%):	6.51					
99.7% Confidence Level k value:	3.00					
Expanded Uncertainty (%):	11.28					

[a] QC Data standard deviation was obtained by averaging the total precisions of each drug from their high and low concentrations from Tables XI, XII, XIII, XIV, XV, and XVI.

V. DISCUSSION

The method as outlined in this paper presents a method to detect Andarine, LGD-4033, and Ostarine in equine serum samples at minimal concentrations. With the relative speed of the liquid-liquid extraction when compared to other extraction methods with more steps, forensic laboratories could expect quicker turnaround times for serum samples containing SARMs. In addition, the high sensitivity of the LC-MS for the SARMs included could improve detection of these compounds in samples that previously would have registered as negative. However, while this method does prove acceptable for the detection of SARMs in equine serum samples, there are limitations which should be explored.

A. **Limitations**

One of the limitations of this work was the scope of the drugs studied. The three compounds in this study, while popular RMs, only represent two of the many classes of compounds that fall under the SARMs umbrella. As SARMs continue to be developed and undergo clinical testing, there is certainty that more SARMs beyond the three studied here will appear in doping control samples in the future. Therefore, this work could be expanded on in future studies by including additional SARMs to prepare for their emergence in doping control samples.

Another limitation of this work was the inability to study metabolites of the selected compounds. Should a horse be doped long enough prior to sample collection, the parent compound may not be detectable in the sample, but metabolites of this parent drug would be present. While past research has isolated metabolites that could be targeted in such testing, without administration to actual horses, the metabolites are difficult to obtain. As previously mentioned, the parent compounds of the drugs Andarine and Ostarine are only

detectable for 12-18 hours post administration in equine plasma samples (Hansson et al., 2016), but other metabolites of these drugs are detectable for extended periods in the serum. In future studies, administration of the drugs to horses would provide samples with these metabolites, and the method could be expanded to include those metabolites.

Matrix effects, as seen in Table III, caused a suppression of signal for both LGD-4033 and Ostarine, while it caused an enhancement of the signal for Andarine. In addition, the process efficiency of Andarine, also from Table III, was above 100%, indicating that a component of the matrix enhances the ionization of Andarine, instead of suppressing ionization as with LGD-4033 and Ostarine. In order to improve the ion suppression of LGD-4033, a modified extraction method that cleans up the samples further could be attempted. However, one of the significant strengths to the current protocol is the simple procedure and relatively short length. Additional cleanup steps would only add and lengthen this process. Despite the observed matrix effects of LGD-4033 and Ostarine in the qualitative data, later quantitative data showed detection of the two drugs at expected levels. Therefore, the matrix effects can be largely ignored.

Finally, the SARM compounds used in this study were all negatively ionizing, thus a negative ionization method was used. In addition, a negative ionization method requires a negatively ionizing standard. Most routine screening methods are created for positive ionization, as most drugs of abuse are positively ionizing. Therefore the choices for a negatively ionizing internal standard were limited. While a deuterated version of a SARM included in this study would have been preferable, none were available for purchase. A deuterated SARM, S1-d4 was purchased to use as an internal standard, but the standard was not stable in water. The internal standard used, butalbital-d5, exhibited greater stability

in water, and when used for concentration analysis, provided the concentrations expected. However, this research would be preferable with a deuterated version of Andarine, LGD-4033, or Ostarine, provided reasonable stability of the standard.

B. **Conclusion**

The extraction method and instrumental technique as presented in this work have proved to be an efficient and successful way to detect Andarine, LGD-4033, and Ostarine in equine plasma samples. By combining the ease of a liquid-liquid extraction with the sensitivity and field's emerging favor towards LC-MS, the protocols outlined would be favorable for implementation in the detection of Andarine, LGD-4033, and Ostarine in equine doping samples. The author believes this to be the first method validation for Andarine, LGD-4033, and Ostarine to combine liquid-liquid extraction with LC-MS QQQ for equine plasma samples.

In the future, the method presented could be expanded to include more SARMs, in order to keep up with doping trends in the horse racing industry. In addition, the administration of the Andarine, Ostarine, and LGD-4033 to actual horses would provide opportunity to validate methods for the detection of metabolites in plasma and possibly urine samples. Should deuterated versions of the aforementioned SARMs become available, replacement of the current internal standard with one of these versions should also be explored. As it stands, the current method is an effective way to detect Andarine, Ostarine, and LGD-4033 in equine plasma samples and is recommended for implementation in routine doping control in order to prevent and catch illicit use of these substances.

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APPENDIX

Acquisition Method Report

Agilent Technologies

Acquisition Method Info

Method Name Blood Negative Ionization Screen.m

Method Path C:\MassHunter\Methods\Blood Negative Ionization Screen.m

Method Description Default Method

Device List

HiP Sampler
Binary Pump
Column Comp.
QQQ

MS QQQ Mass Spectrometer

Ion Source	AJS ESI	**Tune File**	atunes.tune.xml
Stop Mode	No Limit/As Pump	**Stop Time (min)**	1
Time Filter	On	**Time Filter Width (min)**	0.07

Time Segments

Index	Start Time (min)	Scan Type	Ion Mode	Div Valve	Delta EMV	Store
1	0	MRM	ESI+Agilent Jet Stream	To Waste	0	No
2	0.2	MRM	ESI+Agilent Jet Stream	To MS	400	Yes
3	3.5	MRM	ESI+Agilent Jet Stream	To Waste	0	No

Time Segment 1

Scan Segments

Cpd Name	ISTD?	Prec Ion	MS1 Res	Prod Ion	MS2 Res	Dwell	Frag (V)	CE (V)	Cell Acc (V)	Polarity
Compound 1	No	350	Unit/Enh (6490)	200	Unit/Enh (6490)	200	135	0	7	Positive

Scan Parameters

Data Stg	Threshold
Centroid	0

Source Parameters

Parameter	Value (+)	Value (-)
Gas Temp (°C)	300	300
Gas Flow (l/min)	10	10
Nebulizer (psi)	45	45
SheathGasHeater	250	250
SheathGasFlow	8	8
Capillary (V)	3500	3500
VCharging	500	500

Time Segment 2

APPENDIX (continued)

Acquisition Method Report

Agilent Technologies

Scan Segments

Cpd Name	ISTD?	Prec Ion	MS1 Res	Prod Ion	MS2 Res	Dwell	Frag (V)	CE (V)	Cell Acc (V)	Polarity
Andarine	No	440.1	Unit/Enh (6490)	150.1	Unit/Enh (6490)	25	150	20	4	Negative
Andarine	No	440.1	Unit/Enh (6490)	107.1	Unit/Enh (6490)	25	150	60	4	Negative
Andarine-d4	No	405.1	Unit/Enh (6490)	261.1	Unit/Enh (6490)	25	165	16	4	Negative
Andarine-d4	No	405.1	Unit/Enh (6490)	205	Unit/Enh (6490)	25	165	24	4	Negative
Derecoxib	No	396.1	Unit/Enh (6490)	332.1	Unit/Enh (6490)	25	155	20	4	Negative
Derecoxib	No	396.1	Unit/Enh (6490)	302.1	Unit/Enh (6490)	25	155	20	4	Negative
MK-2866	No	388.1	Unit/Enh (6490)	185.1	Unit/Enh (6490)	25	125	40	4	Negative
MK-2866	No	388.1	Unit/Enh (6490)	118.1	Unit/Enh (6490)	25	125	24	4	Negative
LGC-4033	No	383.1	Unit/Enh (6490)	267.1	Unit/Enh (6490)	25	95	8	4	Negative
LGC-4033	No	383.1	Unit/Enh (6490)	45.1	Unit/Enh (6490)	25	95	12	4	Negative
Bumetanide	No	363.1	Unit/Enh (6490)	207.1	Unit/Enh (6490)	25	190	12	4	Negative
Bumetanide	No	363.1	Unit/Enh (6490)	80.1	Unit/Enh (6490)	25	190	16	4	Negative
Meloxicam	No	350	Unit/Enh (6490)	286.1	Unit/Enh (6490)	25	95	4	4	Negative
Meloxicam	No	350	Unit/Enh (6490)	146	Unit/Enh (6490)	25	95	12	4	Negative
Furosemide-d5	No	334	Unit/Enh (6490)	285	Unit/Enh (6490)	25	100	15	4	Negative
Furosemide-d5	No	334	Unit/Enh (6490)	78	Unit/Enh (6490)	25	100	25	4	Negative
5-hydroxydantrolene	No	329	Unit/Enh (6490)	286	Unit/Enh (6490)	25	100	0	4	Negative
Furosemide	No	329	Unit/Enh (6490)	285	Unit/Enh (6490)	25	100	15	4	Negative
5-hydroxydantrolene	No	329	Unit/Enh (6490)	230.1	Unit/Enh (6490)	25	100	8	4	Negative
Furosemide	No	329	Unit/Enh (6490)	78	Unit/Enh (6490)	25	100	25	4	Negative
Meclofenamic Acid	No	294	Unit/Enh (6490)	258.1	Unit/Enh (6490)	25	95	0	4	Negative
Meclofenamic Acid	No	294	Unit/Enh (6490)	214	Unit/Enh (6490)	25	95	12	4	Negative
Fluphenamic Acid	No	280.1	Unit/Enh (6490)	236.1	Unit/Enh (6490)	25	105	12	4	Negative
Fluphenamic Acid	No	280.1	Unit/Enh (6490)	215.1	Unit/Enh (6490)	25	105	28	4	Negative
Modafinic Acid	No	273.1	Unit/Enh (6490)	167.1	Unit/Enh (6490)	25	35	12	4	Negative
Modafinic Acid	No	273.1	Unit/Enh (6490)	49	Unit/Enh (6490)	25	35	0	4	Negative
Butalbital-5	No	228.1	Unit/Enh (6490)	41.9	Unit/Enh (6490)	25	110	8	4	Negative
Ibuprofen	No	205.1	Unit/Enh (6490)	161.1	Unit/Enh (6490)	25	70	0	4	Negative
Ibuprofen	No	205.1	Unit/Enh (6490)	137.2	Unit/Enh (6490)	25	70	4	4	Negative

Scan Parameters

Data Stg	Threshold
Centroid	0

Source Parameters

Parameter	Value (+)	Value (-)
Gas Temp (°C)	300	300
Gas Flow (l/min)	10	10
Nebulizer (psi)	45	45
SheathGasHeater	250	250
SheathGasFlow	8	8
Capillary (V)	3500	3500
VCharging	500	500

APPENDIX (continued)

Acquisition Method Report

Agilent Technologies

Time Segment 3

Scan Segments

Cpd Name	ISTD?	Prec Ion	MS1 Res	Prod Ion	MS2 Res	Dwell	Frag (V)	CE (V)	Cell Acc (V)	Polarity
Compound 1	No	350	Unit/Enh (6490)	200	Unit/Enh (6490)	200	135	0	7	Positive

Scan Parameters

Data Stg	Threshold
Centroid	0

Source Parameters

Parameter	Value (+)	Value (-)
Gas Temp (°C)	300	300
Gas Flow (l/min)	10	10
Nebulizer (psi)	45	45
SheathGasHeater	250	250
SheathGasFlow	8	8
Capillary (V)	3500	3500
VCharging	500	500

Chromatograms

Chrom Type	Label	Offset	Y-Range
TIC	TIC	0	10000000

Instrument Curves

Actual

Name: HiP Sampler **Model: G1367E**

Auxiliary	
Draw Speed	200.0 µL/min
Eject Speed	200.0 µL/min
Draw Position Offset	0.5 mm
Wait Time After Drawing	0.0 s
Sample Flush Out Factor	5.0
Vial/Well bottom sensing	Yes
Injection	
Injection Mode	Injection with needle wash
Injection Volume	30.00 µL
Needle Wash	
Needle Wash Location	Flush Port
Wash Time	3.0 s
High throughput	
Automatic Delay Volume Reduction	No
Overlapped Injection	
Enable Overlapped Injection	Yes
Overlapped Injection Start Mode	After Period Of Time
Overlapped Injection Wait Time	3.25 min
Valve Switching	
Valve Movements	0
Valve Switch Time 1	
Switch Time 1 Enabled	No
Valve Switch Time 2	
Switch Time 2 Enabled	No
Valve Switch Time 3	
Switch Time 3 Enabled	No
Valve Switch Time 4	
Switch Time 4 Enabled	No
Stop Time	
Stoptime Mode	As pump/No limit
Post Time	
Posttime Mode	Off

APPENDIX (continued)

Acquisition Method Report

Agilent Technologies

Name: Binary Pump **Model:** G1312B

Flow	0.500 mL/min
Use Solvent Types	No
Low Pressure Limit	0.00 bar
High Pressure Limit	600.00 bar
Maximum Flow Gradient	100.000 mL/min²
Stroke A	
Automatic Stroke Calculation A	Yes
Stroke B	
Automatic Stroke Calculation B	Yes
Compress A	
Compressibility Mode A	Compressibility Value Set
Compressibility A	40 10e-6/bar
Compress B	
Compressibility Mode B	Compressibility Value Set
Compressibility B	90 10e-6/bar
Stop Time	
Stoptime Mode	Time set
Stoptime	4.00 min
Post Time	
Posttime Mode	Off

Timetable

Timetable

	Time	Function	Parameter
1	0.50 min	Change Flow	Flow: 0.5 mL/min
2	0.50 min	Change Max. Pressure Limit	Max. Pressure Limit: 600.00 bar
3	0.50 min	Change Solvent Composition	Solvent composition A: 55.0 % B:45.0 %
4	1.50 min	Change Flow	Flow: 0.5 mL/min
5	1.50 min	Change Max. Pressure Limit	Max. Pressure Limit: 600.00 bar
6	1.50 min	Change Solvent Composition	Solvent composition A: 0.0 % B:100.0 %
7	2.50 min	Change Flow	Flow: 0.5 mL/min
8	2.50 min	Change Max. Pressure Limit	Max. Pressure Limit: 600.00 bar
9	2.50 min	Change Solvent Composition	Solvent composition A: 0.0 % B:100.0 %
10	2.51 min	Change Flow	Flow: 0.5 mL/min
11	2.51 min	Change Max. Pressure Limit	Max. Pressure Limit: 600.00 bar
12	2.51 min	Change Solvent Composition	Solvent composition A: 55.0 % B:45.0 %

Solvent Composition

	Channel	Solvent 1	Name 1	Solvent 2	Name 2	Selected	Used	Percent
1	A	H2O	H2O 0.2% F.A.	H2O		Ch. 1	Yes	55.0 %
2	B	MeOH	MeOH 0.2% F.A.	H2O		Ch. 2	Yes	45.0 %

APPENDIX (continued)

Acquisition Method Report

Agilent Technologies

Name: Column Comp. **Model:** G1316A

Valve Position	Port 1 -> 6
Left Temperature Control	
Temperature Control Mode	Temperature Set
Temperature	40.00 °C
Enable Analysis Left Temperature	
Enable Analysis Left Temperature On	Yes
Enable Analysis Left Temperature Value	0.50 °C
Right Temperature Control	
Right temperature Control Mode	Not Controlled
Enable Analysis Right Temperature	
Enable Analysis Right Temperature On	Yes
Enable Analysis Right Temperature Value	0.50 °C
Stop Time	
Stoptime Mode	As pump/injector
Post Time	
Posttime Mode	Off

VITA

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