Lipidomics Investigations for Improved Equine Anti-Doping using LC-HRMS

by

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Doctor of Philosophy (Science)

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I, Kathy Tou declare that this thesis is submitted in fulfilment of the requirements for the award of *the Degree of the Doctor of Philosophy (Science)* in the Centre for Forensic Science, School of Mathematics and Physical Sciences (Faculty of Science) at the University of Technology Sydney.

This thesis is wholly my own work unless otherwise referenced or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

This document has not been submitted for qualifications at any other academic institution.

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Abbreviations

% RE	Percent relative error
% RSD	Percentage relative standard deviation
10-HDHA	10-hydroxydocosahexaenoic acid
11-Dehydro TxB_2	11-dehydro thromboxane B_2
11-HEDE	11-hydroxyeicosadienoic acid
11(12)-EET-D_{11}	11,12-Epoxyeicosatreinoic acid - D_{11}
12-HETE	12-hydroxyeicosatetraenoic acid
13-HDHA	13-hydroxydocosahexaenoic acid
13-HODE	13-hydroxyoctadecadienoic acid
14-HDHA	7-hydroxydocosahexaenoic acid
14,15-DiHETE	14,15-dihydroxy-eicosa,5,8,11,17-tetraenoic acid
14(15)-DiHET-D_{11}	14,15-dihydroxyeicosatrienoic acid
15-HEDE	15-hydroxyeicosadienoic acid
15-HEPE	15-hydroxyeicosapentaenoic acid
15-HETE	15-hydroxyeicosatetraenoic acid
15(S)-HPETE	15-hydroperoxyicosatetraenoic acid
17-HDHA	17-hydroxydocosahexaenoic acid
18-HEPE	18-hydroxyeicosapentaenoic acid
20-HDHA	20-hydroxydocosahexaenoic acid
4-HDHA	4-hydroxydocosahexaenoic acid
5-HEPE	5-hydroxyeicosapentaenoic acid
5-HETE	5(S)-hydroxyeicosatetraenoic acid
5-KETE	5-oxo-eicosatetraenoic acid
5-LOX	5-lipoxygenase
5(S)-HPETE	5-hydroperoxyeicosatetraenoic acid
6-Keto $PGF_{1\alpha}$	6-keto prostaglandin $F_{1\alpha}$
7-HDHA	4-hydroxydocosahexaenoic acid
9-HEPE	9-hydroxyeicosapentaenoic acid

9-HETE	9-hydroxyeicosatetraenoic acid
9-HODE	9-hydroxyoctadecadienoic acid
9-HOTrE	9-hydroxyoctadecatrienoic acid
AA	Arachidonic acid
ABP	Athlete biological passport
ACN	Acetonitrile
AEA	Arachidonoyl Ethanolamide
AM404	N-(4-hydroxyphenyl) arachidonylamide
ANOVA	Analysis of Variance
AORC	Association of Official Racing Chemists
ARFL	Australian Racing Forensic Laboratory
BPs	Bisphosphonates
C	Cortisone
CBD	Cannabidiol
CE	Collision energy
CI	Calcium ionophore
COX	Cyclooxygenase
CTX-1	Type-1 Collagen
DCM	Dichloromethane
DDA	Data dependent acquisition
DHA	Docosahexaenoic acid
DIA	Data independent acquisition
EBP	Equine biological passport
ELISA	Enzyme-linked immunosorbent assay
EPA	Eicosapentaenoic acid
EPA	Eicosapentaenoic acid
ERC	Endogenous reference compound
ESI	Electrospray Ionisation
EtOH	Ethanol
FA	Formic acid
FC	Fold change

FLP	Fluticasone Propionate
FWHM	Full width at half maximum
GC-MS	Gas chromatography-mass spectrometry
h	Hour
H_2O	Water
HC	Hydrocortisone
HC/C	Hydrocortisone/Cortisone Ratio
HCl	Hydrochloric acid
HETEs	Hydroxyeicosatetraenoic acid
HMDB	Human Metabolome Database
HPETEs	Hydroperoxyeicosatetraenoic acid
HPLC-UV	High performance liquid chromatography with ultraviolet detection
HRMS	High-resolution mass spectrometry
HyMAS	Hyphenated Mass Spectrometry Laboratory
IFHA	International Federation of Horseracing Authorities
IL-1	Interleukin-1
IPA	Isopropanol
IQR	Interquartile range
IRL	Individual reference limits
IS	Internal Standard
IV	Intravenous
LC	Liquid Chromatograph
LC-MS	Liquid-chromatography mass spectrometry
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
LOX	Lipoxygenase
LPS	Lipopolysaccharide
LTB4	Leukotriene B_4
LTD4	Leukotriene D_4

LTE4	Leukotriene E_4
m/z	Mass to charge
ME	Matrix effects
MeOH	Methanol
MRM	Multiple reaction monitoring
MS	Mass Spectrometry
MS-DIAL 4	Mass Spectrometry-Data Independent Analysis software
MTBE	Methyl tert-butyl ether
MUFAs	Monounsaturated fatty acids
NATA	National Association of Testing Authorities
NF	Nuclear factor
ng/mL	Nanograms per millilitre
NH_4OH	Ammonium hydroxide
NMR	Nuclear magnetic resonance
NSAIDs	Non-steroidal anti-inflammatories
OEA	Oleoyl Ethanolamide
OEA/AEA	Oleoyl Ethanolamide/Arachidonoyl Ethanolamide ratio
PBZ	Phenylbutazone
PCA	Principal component analysis
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGI_2	Prostacyclin
PLA_2	Phospholipase A_2
PLS	Partial least squares
PP	Protein precipitation
PRL	Population reference limits
PUFAs	Polyunsaturated fatty acids
QC	Quality control
QToF-MS	Quadrupole Time-of-Flight mass spectrometry
S/N	Signal to noise ratio

SPE	Solid phase extraction
TA	Tiludronic Acid
TACA	Triamcinolone Acetonide
TCA	Trichloroacetic acid
TEA	Triethylamine
THC	tetrahydrocannabinol
TMOA	Trimethyl orthoacetate
TMSD	Trimethylsilyl diazomethane
TxA_2	Thromboxane A_2
TxB_2	Thromboxane B_2
uL	Microlitres
ZA	Zoledronic Acid

Publications and Conference Proceedings

Journal Publications

1. **Tou, K.,** et al. *Measurements of Hydrocortisone and Cortisone for Longitudinal Profiling of Equine Plasma by LC-MS/MS.* Drug. Test. Anal. 2022; 14(5): 943-952.
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3. **Tou, K.,** et al. *Lipid and Corticosteroid Biomarkers Under the Influence of Bisphosphonates*. Drug. Test. Anal. 2025; 17:1107-1117.

Conference Proceedings/Presentations (presenting author bolded)

1. **Tou K.** Cawley A, Bishop D, Bowen C, Noble G, Fu S. – *Lipidomics for Enhanced Equine Anti-Doping using LC-HRMS.* Poster presentation for Metabolomics 2021 Conference (June 2021), Online.
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3. **Tou K.** Cawley A, Bishop D, Bowen C, Noble G, Labens R, Fu S. – *You can run but you can't go undetected.* Oral presentation for the Australian and New Zealand Society for Mass Spectrometry (ANZSMS) (November 2021), Online.
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7. **Tou K.** Cawley A, Noble G, Loy J, Bishop D, Bowen C, Keledjian J, Sornalingam K, Richards S, Fu S. – *Complementary Biomarker Detection for Nitrogenous Bisphosphonate Use in Racehorses*. Oral presentation at the 60th annual meeting of The International Association of Forensic Toxicologists (TIAFT) conference (August 2023), Rome, Italy.
8. **Tou K.** Cawley A, Noble G, Loy J, Bishop D, Bowen C, Keledjian J, Sornalingam K, Richards S, Fu S. – *The Effects of Lipid and Corticosteroid Biomarkers Under the Influence of a Nitrogenous and Non-Nitrogenous Bisphosphonate.* Oral presentation at the 23rd International Conference of Racing Analysts and Veterinarians (September 2023), Hong Kong SAR, China. – Winner of Young Scientist Award (Best Oral Presentation – Analytical Session)
9. **Tou K.** Cawley A, Bowen C, Keledjian J, Fu S. – *From Reference Population Limits Towards Individual Reference Limits for Equine Anti-Doping*. Oral presentation at the 23rd Triennial Meeting of the International Association of Forensic Sciences (IAFS) in conjunction with the 26th Australian New Zealand Forensic Science Society (ANZFSS) Forensic Science Symposium (November 2023), Sydney, Australia.

Abstract

Lipidomics is a comprehensive yet novel analysis tool for the equine anti-doping field. The development of analytical methods for the analysis of lipids (e.g. eicosanoids associated with inflammation) has been necessary due to the integrity and ethical issues that arise from the use of legitimate therapeutics and mis-use of non-approved anti-inflammatory substances. With the discovery of novel biomarkers for detecting doping practices, the potential exists for a larger number of drugs to be indirectly detected over extended periods of time. With these additional novel biomarkers, these can be included into the equine biological passport, an intelligence based anti-doping program allowing for longitudinal profiling of individual horses.

The arachidonic acid cascade (i.e. eicosanoids) are involved in the inflammatory pathways with the ability to decrease in the presence of anti-inflammatory drugs. A method was developed to monitor 20 lipids, 5 corticosteroids and 14 internal standards. From these targeted biomarkers, 2 lipids and 2 corticosteroids were quantified. The markers of Oleoyl ethanolamide (OEA), Arachidonoyl ethanolamide (AEA), hydrocortisone (HC) and cortisone (C) were quantified using equine plasma from 750 pre-race blood samples to determine basal concentrations. In agreement with previous work and literature demonstrating consistency in levels of C, the concentrations using this method showed consistency allowing for the proposal of the HC to C ratio. The following respective upper and lower limits were proposed: OEA, 5.6 ng/mL and 0.21 ng/mL, AEA: 0.6 ng/mL and 0.2 ng/mL, HC: 200 ng/mL and 0.02 ng/mL, C: 10 ng/mL and 1.0 ng/mL and HC/C: 50 and 0.12.

With an understanding of the basal levels for specific biomarkers, this knowledge was applied to four separate administration studies: 2 corticosteroids and 2 bisphosphonates. Exogenous corticosteroids have long been used in the equine racing industry to treat inflammation and whilst many are legitimate therapeutics, these are prohibited during competition. The 2 corticosteroids studied in this project were triamcinolone acetonide (TACA) and fluticasone propionate (FLP). Administration of these corticosteroids caused endogenous fluctuations within each individual horse allowing for the biomarkers of HC, HC/C and OEA giving extended indirect detection in comparison to the targeted parent compound.

The bisphosphonate administrations of tiludronic acid and zoledronic acid is of significance due to the growing use of this class of compounds to treat bone disorders in the horse. At present, there are only two non-nitrogenous bisphosphates approved for the treatment of Navicular syndrome in the horse (tildronate and clodronate) with no nitrogenous bisphosphonates approved for use. From the bisphosphonate administration studies, specific biomarkers were able to provide evidence of a non-nitrogenous bisphosphonate whilst others provided evidence of a nitrogenous bisphosphonate. The biomarkers for indicating non-nitrogenous bisphosphonates include $PGF_{2\alpha}$, 18-HEPE and the 15(S)/5(S)-HETE ratio. Comparatively, the biomarkers of HC and HC/C provided evidence of a nitrogenous bisphosphonate.

Further work translating the research method using the routine analysis method was necessary to allow for a better understanding of these biomarkers using an accredited plasma method at the Australian Racing Forensic Laboratory. From the routine analysis method, upper and lower thresholds of 0.67 ng/mL and 0.12 ng/mL for AEA and 5.5 ng/mL and 0.02 ng/mL for OEA. Nonetheless, future work will need to include international collaboration with other laboratories to expand and corroborate the findings presented in this work. In addition, the monitoring of additional biomarkers and/or their ratios in the lipid pathways could provide greater confidence for interpretations of a misuse of a doping agent compared to natural variation.

Chapter 1: Introduction

Rationale

This introduction provides the general background information necessary to understand the foundations of this PhD project. It was designed to give an overview of biomarkers (specifically eicosanoids), the current state of equine anti-doping and the different components necessary for a lipidomics workflow. This includes possible instrumentation, data acquisition and analysis methods that lipidomics workflows can follow. In addition, a section is also dedicated to studies already in literature that focus on the use of eicosanoids for equine anti-doping screening for certain classes of administrations. This includes exogenous corticosteroids, non-steroidal anti-inflammatories (NSAIDs), and cannabidiol. There is also a small section dedicated to the potential for lipidomics to contribute to longitudinal assessments for equine anti-doping.

This chapter (section 1.1-1.8) was taken from a first author publication titled “Towards Non-Targeted Screening of Lipid Biomarkers for Improved Equine Anti-Doping” published in *Molecules.* The preparation of the initial manuscript was performed by K. Tou with edits provided by A. Cawley, C. Bowen, D. Bishop, and S. Fu.

Towards Non-Targeted Screening of Lipid Biomarkers for Improved Equine Anti-Doping

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1.1 Biomarkers for Equine Anti-Doping

The current approach to equine anti-doping is focused on the targeted detection of prohibited substances [1]. However, as new substances are rapidly being developed, the need for complimentary methods of monitoring is important to ensure the integrity of the racing industry is upheld [1]. The use of biomarkers for the detection of doping abuse is a significant advancement for sports anti-doping. Teale *et al* [2]. define biomarkers as an "individual biological parameter or substance (metabolite, protein or transcript); the concentration of which is indicative of the use or abuse of a drug or therapy". With the discovery of novel biomarkers for detecting doping abuse, the potential exists for a larger number of drugs to be indirectly detected and over longer periods of time. However, with indirect detection, there is the possibility of the method not being specific and the increased likelihood of inconsistent results [3]. This review will focus on the use of lipidomics for indirect detection of substances in equine racing contributing to an intelligence-based anti-doping strategy [4].

1.2 Current Challenges for Targeted Screening

Doping practices are increasingly sophisticated for all sports with testing laboratories consistently improving their workflows to identify the ever-growing list of prohibited substances. Current detection methods, however, are by nature limited in scope to a defined number of substances and the applicability of the analytical technique used [5]. Maintaining a contemporary scope of testing makes direct detection continually difficult due to availability of reference materials. An "omics" approach may provide an alternative to direct detection of doping [5,6]. The use of metabolomics has been utilised in many different laboratories to measure metabolites at low levels relative to time-related biological responses of a drug administration [3,5,6]. This provides a framework for non-targeted detection, particularly for drugs that have a short half-life but long lasting effect on any individual system [5].

1.3 Lipids

Lipidomics is a growing field involved in the characterisation of lipids, their function and metabolism in a biological system [7,8]. Lipids are non-polar molecules with diversity of chemistry and functionality [9,10]. In conjunction with carbohydrates, lipids are the main energy source for equine striated muscles [11]. There are a number of different classes of lipids including monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) [12,13]. MUFAs are lipids that have a single double bond present in the compound and usually only exist in seeds or marine organisms however, are naturally rare [12]. PUFAs comparatively contain more than one double bond, are more commonly found [12] and have various biological effects including platelet aggregation and inflammation [14]. In animals, common PUFAs include arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) [12]. The most relevant and important oxygenated products for the racing industry are lipids known as the eicosanoids [12]. Eicosanoids are a large subclass predominately defined by the 20 carbon chain containing over 100 lipid mediators including prostaglandins, thromboxanes, leukotrienes, hydroxy fatty acids and lipoxins [12,15] with the majority derived from AA, an omega-6 fatty acid [16]. Eicosanoids are believed to act as inflammatory mediators since they have the ability to mimic inflammatory symptoms and decrease in the presence of anti-inflammatory drugs [8,14]. Disruption of eicosanoids can cause a range of inflammatory

pathological conditions including asthma, chronic obstructive pulmonary disease, fevers, pain, a range of cardiovascular diseases and cancers [15]. Eicosanoids are synthesised at the site of injury in order to control and regulate the inflammatory response [7].

AA is released from membrane phospholipids through the activation of phospholipase A_2 enzyme (PLA_2) [14]. It can be further converted into other eicosanoids in the cascade (Figure 1.1). In a non-targeted sense, it should be possible to utilise the AA cascade to determine which lipids are being affected following a drug administration. This cascade includes prostaglandin D_2 (PGD_2) [17], prostaglandin E_2 (PGE_2) [18], prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) [7], thromboxane B_2 (TXB_2) [19], 11-dehydro thromboxane B_2 (11-Dehydro TXB_2)[20], 6-keto prostaglandin $F_{1\alpha}$ (6-Keto $PGF_{1\alpha}$) [21], 15(S)-hydroxyeicosatetraenoic acid (15-HETE) [7], 5(S)-hydroxyeicosatetraenoic acid (5-HETE) [22], leukotriene B_4 (LTB_4) [23-25], leukotriene D_4 (LTD_4) [23,25] and leukotriene E_4 (LTE_4) [23,25]. The analogues of AA are also of interest including arachidonoyl ethanolamide (AEA) [26,27] and oleoyl ethanolamide (OEA) [26].

Figure 1.1: Arachidonic Acid Cascade, adapted from various sources [7,8,14,17-29]

Abbreviations of eicosanoids: arachidonoyl ethanolamide (AEA), oleoyl ethanolamide (OEA), prostaglandin (PG), thromboxane (Tx), hydroperoxyeicosatetraenoic acids (HPETES), hydroxyeicosatetraenoic acid (HETE) and leukotriene (LT).

AEA is an endogenous cannabinoid ligand that has binding activity resulting in pharmacological effects of tetrahydrocannabinol (THC) such as euphoria and calmness [26,27]. In a variety of cells, cannabinoid agonists have caused an increase in the amount of AA [26]. This has been hypothesised to result from the combination of PLA_2 and acyltransferase inhibition [26]. AA can also be converted into N-(4-hydroxyphenyl) arachidonylamide (AM404) which displays analgesic properties and the ability to lower body temperature [28,29]. AM404 is

produced when acetaminophen is metabolised in the body to produce *p*-aminophenol, which is then conjugated with AA [29]. AM404 has been reported to inhibit the cyclooxygenase (COX) pathways leading to the decreased formation of PGE_2 demonstrating effectiveness as a COX-2 enzyme inhibitor to reduce the production of prostaglandins by consumption of AA [29]. The COX and lipoxygenase (LOX) pathways are of particular interest for equine anti-doping due to their augmentation following anti-inflammatory treatments.

The prostaglandins are monocarboxylic acids with two side chains at carbon 7 and 8 attached to a central, five-membered ring [14]. Prostaglandins have oxygen-containing substituents in various positions in the molecule with naming of the prostaglandins ranging from PGA to PGI depending on the basis of the substituents in the ring and further sub-grouped into three series depending on the degree of unsaturation [14]. Prostaglandins are one of the key compounds in the generation of the inflammatory response due to increase in concentrations in inflamed tissue [30]. Prostaglandins are formed from AA being converted by the COX enzyme to prostaglandin G_2 and H_2[17]. Prostaglandin endoperoxide-D-isomerase can convert PGH_2 into a mixture of PGD_2, PGE_2 and $PGF_{2\alpha}$ [17]. PGH_2 can also produce prostacyclin (PGI_2) that may further metabolise to a more stable compound, 6-keto $F_{1\alpha}$ [19]. PGEs and PGIs have been identified to mimic the signs of inflammation caused by vasodilation and swelling due to increase in vascular permeability [14].

Prostaglandins can also convert into thromboxane A_2 (TXA_2), an unstable intermediate in the production of TXB_2, which further metabolises to form 11-dehydro TXB_2[20]. Thromboxanes are major products of prostaglandin endoperoxides in platelets, lungs and the spleen [14]. The production of new platelets could potentially have a high capacity to further increase the synthesis of TXA_2, leading to increased amount of TXB_2 and 11-dehydro TXB_2 [19]. The use of non-steroidal anti-inflammatory drugs (NSAIDs) result in PGI_2 and thromboxane synthesis being inhibited [19].

Using the LOX pathways, AA converts into esterified hydroperoxyeicosatetraenoic acids (HPETEs) [31]. HPETEs are further reduced to their corresponding hydroxyeicosatetraenoic acids (HETEs) [7]. For example, using the 15-LOX enzyme, AA will metabolise into 15-HPETE and then further metabolises to 15-HETE. Similarly, the 5-LOX enzyme converts AA to 5-HPETE and

further to 5-HETE. The leukotrienes are an oxygenated metabolite of polyunsaturated fatty acids, but the initial formation is catalysed by lipoxygenases [23]. Leukotrienes are produced from AA using the 5-lipoxygenase (5-LOX) enzyme to produce Leukotriene A_4 (LTA_4), an unstable epoxide. LTA_4 is hydrolysed to LTB_4 or conjugated with a glutathione to yield Leukotriene C_4 (LTC_4) and its metabolites LTD_4 and LTE_4. Leukotrienes are known for their strong vascular effect with the most effective being LTB_4 in comparison to LTC_4 and LTD_4 [14,15]. In the presence of more leukocytes, the leukotrienes also have a role in the inflammatory process by increasing blood pressure [23].

1.4 Analytical Techniques for Lipidomics

1.4.1 Sample Preparation

With a wide range of analytical techniques applicable for doping analysis, optimised sample preparation methods have been developed [32]. There are typically two types of sample preparation strategies: protein precipitation (PP) which is monophasic; and lipid extractions which are biphasic [33]. For the latter solid phase extraction (SPE) and liquid-liquid extraction (LLE), are commonly used [32,33]. LLE efficiency is improved if a mixture of solvents is used in comparison to only a single solvent [34]. SPE is a useful sample preparation method as it allows for the preconcentration of low sample volumes, which provides sensitive detection [35].

Chambers *et al* .[32] conducted a study comparing the various sample preparation methods including PP, SPE and LLE to optimise sample preparation for the analysis of eicosanoids. While PP was rapid and simple, it doesn't often provide a clean extract for analysis, however the type of organic solvent used in the PP did influence the cleanliness of the extract. The MeOH extract contained 40% more potentially interfering phospholipids in comparison to the ACN extract. This result is consistent with the work of Bruce *et al*. [36] and Stojiljkovic *et al.* [5] showing that ACN or acetone was the best solvent for PP-based methods. ACN is more efficient in protein removal due to the higher dielectric constant and lower viscosity it possesses [5]. Ammonium sulphate can also be used, however it has to be combined with SPE or a secondary LLE to ensure a cleaner extract before liquid-chromatography mass spectrometry (LC-MS) analysis [5]. SPE stationary phases are selected along similar principles to

chromatographic column phases, with the size, polarity, and charge of analyte considered. Pure cation exchange stationary phase have produced cleaner extracts however, polymetric mixed-mode strong cation exchange can enable hydrophobic phospholipids containing two alkyl chains and lysophospholipids to be removed more efficiently. In comparison, LLE has been widely investigated and provides a cleaner method of sample preparation in comparison to PP [32]. The cleanliness of the extracts were comparable with cation-exchange mixed-mode SPE using three methods; a 3:1 ratio of methyl tert-butyl ether (MTBE) to human plasma, a 3:1 ratio of basified MTBE (5% NH_4OH in MTBE) to human plasma and basified MTBE in a two-step extraction [32].

1.4.2 Analysis

A broad range of analytical techniques can be used to separate, detect and quantify eicosanoids including high performance liquid chromatography with ultraviolet detection (HPLC-UV) [37,38], enzyme-linked immunosorbent assay (ELISA) [39], nuclear magnetic resonance (NMR) [5], gas chromatography-mass spectrometry (GC-MS)[40] and LC-MS [15,39]. The main disadvantage of using HPLC-UV is the limited sensitivity and specificity of UV detection in complex biological matrices such as urine or plasma [15,37,38]. There is also the possibility of the lack of active chromophores in lipids that absorb UV light at the appropriate wavelengths [38]. ELISA is often limited to one analyte per assay and due to eicosanoids having a large number of isomers, there is the possibility of high cross-reactivity affecting quantification [39]. The use of NMR has its advantages as a non-destructive analysis involving minimal sample preparation, however the main disadvantage is the low sensitivity in comparison to GC-MS or LC-MS and the relatively large sample requirement [5]. GC-MS boasts high sensitivity and resolution of eicosanoids that have numerous isomers, however complex sample preparation and derivatisation is usually required [40]. One of the most commonly used LC-MS instruments used is triple quadrupole mass spectrometry due to the high sensitivity and specificity obtained using multiple reaction monitoring acquisition for extremely low level lipids in the equine system [7]. With this high sensitivity, quantitation of eicosanoids have been possible which is further discussed in chapter 1.6 of this review.

The use of high-resolution mass spectrometry (HRMS) in metabolomics has enabled the indirect detection of substances that have a short-half-life but long-lasting effects on biological systems [5]. Following this approach, studies have utilised LC-HRMS for lipidomics to investigate the potential to distinguish isomeric lipids [41]. Lipidomic studies are increasingly using LC-HRMS due to high sensitivity, mass accuracy, resolution and acquisition rates providing the ability to detect subtle differences in complex biological matrices such as plasma and urine [42-45]. The two types of HRMS instruments include the Quadrupole Time-of-Flight mass spectrometry (QToF-MS) and the Orbitraps. These two HRMS instruments have the ability to achieve a mass accuracy of below 5 ppm for higher accuracy in the identification of compounds with some instruments being advertised to have the ability to achieve less than 1 ppm difference [46,47]. Commercially available QToF instruments have resolving power between 35,000 and 70,000 full width at half maximum (FWHM) whilst comparatively, the Orbitraps have over 1,000,000 FWHM [47-49]. Whilst the Orbitraps do outperform the QToFs in mass resolving power, the disadvantage would be the longer accumulation times, which result in the Orbitraps being less suitable for accurate quantification of compounds with narrow LC peaks [46]. Therefore, the identification of unknown lipids using LC-HRMS slightly difficult due to the isomeric and isobaric nature of lipids, but the accuracy of these instruments has the potential to narrow the search to a particular class of lipids rather than the individual compound. The use of LC-HRMS allows for a complementary targeted/non-targeted approach to detect new and emerging drugs that could potentially be used as doping agents and the metabolic signature that can result from such [5].

1.4.3 Data Acquisition Modes

Non-targeted lipidomics workflows allow for the generation of new hypotheses for supporting evidence in biological interpretations or for complementary 'omics' data. Targeted lipidomics is often used for the validation of biomarkers following the untargeted discovery phase or whilst trying to measure certain lipids that are associated with disease or are present in low abundance [50].

Two common modes of data acquisition for LC-HRMS analyses supporting lipidomic workflows are data dependent acquisition (DDA) and data independent acquisition (DIA) [47].

DDA utilises precursor ions that have exceeded an abundance threshold, predefined isotopic pattern, and the presence of diagnostic product ions [47,48]. These ions, once detected, facilitate the acquisition of MS2 data, however, this is limited to ions of relatively high intensity [51]. There is also the possibility of precursor ions and relevant analytes being missed [52]. DDA is well suited to targeted screening, however reproducibility and variation in spectral databases often results in unknown metabolites being difficult to interpret [47].

Comparatively, DIA or sequential window acquisition of all theoretical fragmentation ion (SWATH) allows for simultaneous screening of all precursor ions and their fragmentation pattern within a specified mass to charge (*m/z*) window regardless of intensity [47-49,51,53,54]. The use of DIA for lipidomics was demonstrated after its potential was seen in proteomics [50]. DIA has been shown to be an effective means to produce a larger number of quantifiable results in short time periods with fewer errors relating to reproducibility across replicate analysis [55]. The freedom of ion choices reduces the need for data reacquisition due to unexpected ion formation or unrecognisable compounds [52]. Therefore, with the ability to screen multiple precursors, it is often used for untargeted screening. The major disadvantage of DIA is the potential for the incorrect precursor ion being identified as there is no criteria for ions to undergo dissociation [43]. It is also possible to get a mixture of (i.e. chimeric) spectra due to the wide scanning range of the *m/z* windows [43]. Therefore, in lipidomics, with a large number of isomers and levels of unsaturation, the ability to separate between lipid classes is more difficult [52]. Class separation for lipids is generally limited to DDA workflows where the ability to isolate unit mass would be more appropriate[52]. At the time of writing this review, the authors have identified a gap in the research where there is a lack of research focusing on the use of DIA on eicosanoids specifically. There is research completed on lipids as an entirety focusing on whole classes but lacking on eicosanoids due to the vast number of lipid isomers and levels of unsaturation [52,56]. Another limitation of DIA is the possibility of isomeric or isobaric compounds being indistinguishable due to co-elution with an increased possibility of incorrect compound assignment as these compounds can act as interferences [43]. There is also the lack of spectral databases available for non-targeted screening as most databases utilise DDA [48].

1.5 Statistical Analysis

A comprehensive lipidomics study of molecules in any biological system can result in a large amount of data generated. There are two common approaches to process lipidomic data: chemometric and quantitative [57]. Chemometric analysis is performed on spectral patterns or signal intensity data to identify any lipids through non-targeted screening. Chemometric analysis can be automated, however there is the uncertainty of strict sample uniformity and repetition. Quantitative analysis in comparison is more applicable to biological studies as it allows for the identification of all lipids proceeding to the analysis of the lipid data.

The information collated from quantitative studies are either univariate or multivariate depending on the study being conducted. Saccenti *et al.* discuss the advantages and disadvantages of univariate and multivariate analysis of metabolomics data [58]. Univariate analysis is performed when only one variable is analysed, and it includes methods to test different sets of samples such as ANOVA or t -tests. There are, however, disadvantages with univariate analysis with higher possibilities of equivocal findings due to the requirement for multiple testing corrections. Univariate methods are usually the preferred choice of statistical analysis for biologists due to the ease of interpretation the analysis provides. In comparison, multivariate analysis consists of data that have two or more variables. There are many tools available for multivariate analysis including correlation analysis and simple linear regression which can analyse a data set that contains several hundred or more variables simultaneously, thereby eliminating the need for multiple univariate methods. Multivariate statistical analysis provides advantages that univariate may not including the ability of independent variables to complement each other for the prediction of a dependent variable. Lipidomic studies are often multivariate in nature due to a large number of compounds investigated, however it is also common practice for both univariate and multivariate approaches to be used in a complementary manner [58].

Data is often analysed using both unsupervised and supervised strategies [42]. Unsupervised studies allow the discovery of groups or trends in the data with the most common tool being principal component analysis (PCA). Supervised methods are then often used to discover new biomarkers with partial least squares (PLS) being one of the most common. PLS is a

multidimensional method that utilises a data matrix containing independent variables and relates it to dependent variables [42]. As biomarker research usually involves multiple approaches, it is recommended to combine both unsupervised and supervised methods [58]. Various statistical software programs have been utilised to implement supervised and unsupervised methods. One such software program is the Mass Spectrometry-Data Independent Analysis software version 4 (MS-DIAL 4), where a non-targeted lipidomics platform has been utilised to provide a comprehensive lipid database [59]. This contains retention times, mass-to-charge ratios, isotopic ions, adduct information and other mass spectral information for the possible identification of unknowns and lipid pathways [59]. However, there are many limitations that arise with trying to determine the putative structure of an unknown given the limited information provided from statistical analysis [1,60,61]. Confirmations of putative biomarkers is difficult as comparisons need to be made with authentic reference standards if possible, if not, there is the possibility of custom synthesis but these are highly cost inefficient and may incur timely delays for a highly purified sample [60]. The use of these statistical analyses will help provide the information required to further improve current routine testing using non-targeted workflows to monitor for new biomarkers indicative of doping.

1.6 Studies monitoring eicosanoids for equine anti-doping screening.

The administration of corticosteroids and NSAIDs to alleviate pain and inflammation is known to affect the amount of AA, with the COX and LOX pathways subsequently reducing the concentration of eicosanoids present [62-67]. The use of approved corticosteroids and NSAIDs, whilst being legitimate therapeutics for racehorses out-of-competition, are controlled for race day competition. Therefore, the monitoring of terminal compounds in the AA cascade may prove beneficial for the detection of these prohibited substances. Glucocorticoids are potent anti-inflammatory agents that increase the tolerance for pain which may allow for horses to compete under conditions which could compromise the health of the individual horse and safety of riders. Early studies on eicosanoids were performed using ELISA and high-performance liquid chromatography however, these techniques are known to lack specificity [7]. Currently, the advanced technology of hyphenated mass spectrometry such as triple

quadrupole mass spectrometry and QToF instruments have enabled an enhanced scope of eicosanoid monitoring [7] for targeted screening however, the use of non-targeted methods hasn't been extensively explored. Therefore, there is potential in future studies for untargeted methods to provide evidence of an exogenous administration.

1.6.1 Lipid screening in equine samples

Jackson *et al.* [18] was one of the first studies to explore the use of lipids (specifically PGE_2) in lipopolysaccharide (LPS)-stimulated blood for a comprehensive NSAID screening tool using ELISA. However, there was a moderate degree of variability between the different horses tested due to the LPS-stimulation. Even so, PGE_2 production was less than 50% between 8- and 12-hours post-administration. Therefore, it was proposed that an LPS-stimulated plasma concentration of PGE_2 of less than 500 pg/mL could potentially indicate the administration of an NSAID given the terminal position of PGE_2 in the cascade.

Nolazco *et al.* [11] studied the detection of lipids in racehorses before and after supramaximal exercise utilising an LC-QToF-MS. A non-targeted approach discovered 933 lipids present in the plasma of which 130 were known based on library matches. One-tenth (i.e. 13 out of 130) were deemed statistically different compared to baseline concentrations. From these, three unsaturated fatty acids and six phospholipids displayed an increase in signal intensity, whilst four saturated fatty acids (including n-eicosanoid acid) and five triacylglycerols had a decrease in signal intensity. Various hypotheses were proposed to explain the results which included that during exercise, lipolysis causes the ratio of unsaturated to saturated fatty acids for triacylglycerols contained in adipose tissue to be higher than non-esterified fatty acids. Another possibility is during exercise the use of saturated fatty acids as an energy source is preferred over unsaturated fatty acids, causing an increase in the concentration. Whilst this study provided information about the lipidome changes that occur during exercise in racehorses, limitations include the use of a treadmill rather than a racetrack and the small sample size due to the availability of the horses. This study demonstrated that non-targeted lipid profiling aimed at detecting anti-inflammatory drug use needs to account for the effects of exercise.

1.6.2 Lipid inflammatory markers and their effect from a corticosteroid administration

Corticosteroids including dexamethasone, triamcinolone acetonide (TACA) and flumetasone are commonly used throughout the racing industry to alleviate symptoms of inflammation and prevent further tissue damage caused by inflammatory markers[64].

Mangal *et al.* [7] and Knych *et al.* [64] explored the use of dexamethasone on the effects of the inflammatory mediators. Mangal explored 25 eicosanoids in equine plasma using stable isotope dilution reversed-phased LC-MS whilst Knych utilised triple quadrupole mass spectrometry to monitor six eicosanoids. Both Mangal and Knych employed calcium ionophore (CI) A23187 or LPS-simulation of whole blood, whilst Mangal also used AA exogenously added, to determine whether extra enzymatic activity would increase the production of eicosanoids. The use of CI allows for any free arachidonic acid to be released from surrounding cellular membranes to produce eicosanoids through either the COX or LOX enzyme activity [62,63]. The use of LPS-stimulation allows for the modelling of the COX-enzymes for the production of prostaglandins and thromboxanes from arachidonic acid [30,62,63]. Mangal concluded from the 25 monitored eicosanoids, 9 resulted in reduced levels [7]. Comparatively, Knych concluded that whilst dexamethasone doesn't have a direct effect on the COX-1 enzyme, the effect could be seen on the COX-2 enzyme due to LPS stimulation [64]. Knych also observed that the LOX pathways were affected specifically 5-LOX due to the nuclear-factor (NF) kappa beta enzyme which is a known inducer of the 5-LOX enzyme [64]. From CI-stimulation, significant down-regulation was observed for four eicosanoids, while LPS-stimulation showed five eicosanoids to be significantly down-regulated [64].

TACA is a commonly used, long-lasting and potent glucocorticoid that is known to bind to glucocorticoid responsive genes. This can result in the increase concentrations of anti-inflammatory mediators whilst decreasing the inflammatory markers as demonstrated by another study conducted by Mangal *et al.* using a triple quadrupole mass spectrometer [62]. CI and LPS stimulation were again used to explore the inflammatory mediators in equine plasma. For CI-stimulation, a significant increase for two inflammatory mediators was observed but only one proceeded to show a significant decrease. Comparatively for LPS-stimulation, four

eicosanoids showed significant reduction in concentration then a significant increase in concentration prior to a return to basal levels. This study concluded that major products expressed through the COX-2 enzymatic activity are down-regulated under the influence of an external glucocorticoid suggesting inhibition of the COX-2 enzyme [62]. It is worth noting that there is speculation that PGE_2 has the potential to have a binary role in inflammation given it is produced using the COX-1 enzyme as an inflammatory mediator, however delayed production of PGE_2 by the m-PGES-1 enzyme, coupled to COX-2 gives PGE_2 its anti-inflammatory properties [62,68].

Flumetasone is a potent corticosteroid often used in the treatment of performance related injuries associated with strenuous exercise [69]. Knych *et al.* explored the effects of flumetasone on the inflammatory markers under the stimulation of CI and LPS using a triple quadrupole mass spectrometer. The authors concluded that flumetasone had a direct inhibitory effect on the COX and LOX enzymes, however further study will be required for the specific effect on the 15-LOX enzyme[69]. This was demonstrated with significant change for two eicosanoids in the 5-LOX enzymatic pathway following CI-stimulation [69]. Comparatively utilising LPS-stimulation, six eicosanoids displayed significant reduction indicating a direct effect on the COX enzyme [69].

1.6.3 Lipid inflammatory markers and their effect from an NSAID administration

NSAIDs are known to inhibit predominately the COX-2 enzyme rather than COX-1 [65]. Cuniberti *et al.* utilised several NSAIDs; namely eltenac, naproxen, tepoxalin, SC-560 and NS 398 to study their effects on both COX-1 and COX-2 enzymes using a chromatographic assay [65]. This study concluded that certain NSAIDs (e.g. SC-560, NS 398 and eltenac) can act as either COX-1 or COX-2 enzyme inhibitors whilst other NSAIDs (e.g. tepoxalin and Naproxen) can act as dual inhibitors [65]. This was seen with an *in vitro* model demonstrating 100% inhibition of 3 eicosanoids by all drugs whilst *ex vivo* models also showed signs of inhibition but not to the same extent.

Phenylbutazone (PBZ) is one of the most commonly used NSAIDs in equine medicine for the treatment of training and performance related injuries [63]. The use of PBZ is highly regulated

due to the potential to mask injuries during pre-race (i.e. fitness-to-race) examinations [63]. Knych *et al.* explored how PBZ affected the biomarkers of inflammation following intravenous (IV) and oral administration whilst also utilising stimulation with CI and LPS [63]. The technique used for this study was a triple quadrupole mass spectrometer. The duration of monitored effects of PBZ exceeded the direct detection of PBZ in plasma [63]. Utilising CI-stimulation, there was a significant change for two eicosanoids whilst LPS-simulation showed four eicosanoids to significantly change indicating PBZ is an effective inhibitor of the COX-1 enzyme [63].

1.6.4 Lipid Inflammatory markers and their effect from a cannabidiol administration

Cannabidiol (CBD), a known antidote for inflammation in humans has recently been of concern with growing interest from horse trainers considering the use of hemp-based supplements [70]. The effects of CBD on the equine system have not been extensively studied, however it is believed that there are multiple mechanisms that CBD follows which potentially impact the endocannabinoid pathway, including inflammatory, analgesia and stress responses [70]. There is a hypothesis that CBD inhibits COX and LOX enzymes which decrease the amount of pro-inflammatory eicosanoids that are produced during the inflammatory response [70]. This hypothesis follows a similar mechanism for NSAIDs which have been demonstrated to affect the inflammatory pathways. Ryan *et al.* recently evaluated the anti-inflammatory effects of CBD with results indicating both COX-1 and COX-2 enzymes were being stimulated [70] utilising an Agilent 1260 chromatography system coupled to a triple quadrupole mass spectrometer . Whole blood samples were stimulated using either CI or LPS to monitor the inflammatory mediators. During LPS-stimulation, four eicosanoids were observed to have significant change indicating the effects of both COX-1 and COX-2 enzymes, however there is also evidence of CBD being dose dependent with a higher dose resulting in stronger enzyme inhibition [70]. During CI-stimulation, three eicosanoids following the LOX pathways were affected [70]. The major limitation with this study is the endogenous levels of these inflammatory mediators are not known since the study used an *ex vivo* model of inflammation.

1.7 Potential for lipidomics to contribute longitudinal assessments for equine anti-doping.

The primary focus of anti-doping analyses is the detection and quantification of exogenous drugs or substances in biological matrices such as urine and plasma [4]. However, issues such as the clear distinction between the endogenous and exogenous origin of naturally occurring substances and varying individual levels of biomarkers has been evident for decades. Therefore, the need for a longitudinal perspective is necessary given that it can also be an indicator of pharmaceutical manipulation. The equine biological passport (EBP) can provide a basis for complementing targeted analysis (detecting prohibited substances) with the non-targeted monitoring of biomarkers which may be present after the original substance has been eliminated, metabolised or excreted [71]. The establishment of individual reference limits has been useful in monitoring levels of endogenous biomarkers to determine their respective basal levels, which may differ from the general population. This form of monitoring allows for an indirect approach to monitor of biomarkers in order to potentially increase the detection window of administered drugs. The EBP has dual purposes; allowing for the possible detection of novel doping agents together with directing the resources of racing authorities to participants that may be engaging in prohibited practices [71]. It also allows for the monitoring of substances considered relevant to integrity but which do not have an agreed international threshold that exists to control specific misuse [71]. The EBP can provide a deterrent to the use of prohibited substances, since it has the potential to measure the biological effect of an administered substance for longer periods than the presence of the substance itself [71]. As outlined in this review, a lipidomic component may provide a useful contribution to an EBP. For future work, it would be ideal to include lipids into the EBP based off of lipids that were identified from complementary targeted and untargeted research. To the authors knowledge at the time of writing this review, this potential has not been presented before highlighting the novelty of the passport and the use of lipids throughout the passport.

1.8 Conclusion

In conclusion, the improved use of biomarkers for a complementary targeted/non-targeted approach has been explored to potentially extend the time of detection for an administered substance. The current issues involving the use of targeted metabolomics and lipidomics such as short detection windows and matrix effects requires investigation of non-targeted screening strategies. Lipidomics is an expanding field within the broader area of metabolomics focusing on the lipidome in any system with common polyunsaturated fatty acids being a starting point in the potential for a non-targeted approach. Whilst current research still utilises targeted methods to monitor biomarkers as seen in the various equine studies (including corticosteroids, NSAIDs and cannabidiol), there is considerable potential for non-targeted studies in the future. This can be attributed to improved sample preparation in conjunction to the increased use of high-resolution accurate mass spectrometry for non-targeted methods. The use of DIA and DDA to monitor for biomarkers not already currently monitored, in addition to more sophisticated statistical analysis, will likely realise the potential for non-targeted lipidomic studies in the equine racing industry.

1.9 Endogenous Corticosteroids

Endogenous corticosteroids (or cholesterol-based lipids) have been monitored in the equine racing industry for many years. Corticosteroids are steroid hormones produced in the adrenal glands that bind with glucocorticoid receptors to decrease pro-inflammatory genes whilst increasing expression of anti-inflammatory genes[72]. As glucocorticoids have a hormonal and lipophilic nature, they also have the ability to pass through cell membranes [72]. Commonly monitored endogenous corticosteroids including hydrocortisone and cortisone however, other endogenous corticosteroids including 18-oxocortisol, 18-hydroxycortisol and 11-oxocortisol have the potential to exhibit similar pharmacological effects to hydrocortisone and cortisone in the presence of an exogenous corticosteroid[72].

Hydrocortisone (Refer to figure 1.2 for the structure) (commonly known as cortisol), is a primary glucocorticosteroid that is secreted by the adrenal cortex[73,74]. It is often used by the equine system to limit the response to stress, reduce inflammation and increase the

availability of glucose to induce the feeling of euphoria [75]. Therapeutic corticosteroids are all synthetic deviations of hydrocortisone and there is the potential for abuse in thoroughbred racing[73,76,77].

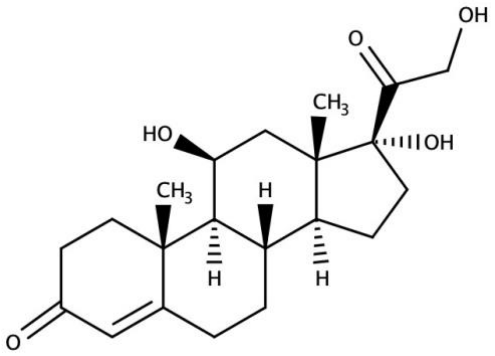

Figure 1.2: Structure of hydrocortisone

The current international threshold for hydrocortisone is only established in urine at 1 µg/mL[78,79]. However, none such threshold exists at the present moment for equine plasma. It must also be noted that there are limitations on the interpretation of hydrocortisone levels in racehorses due to the circadian rhythm observed between morning (peak concentrations) and night (lower concentrations)[80]. A study conducted by Held *et al.* observed that following a dexamethasone (exogenous corticosteroid) administration, the circadian rhythm has influence over the period required prior to hydrocortisone returning to basal levels[80]. Such variation in hydrocortisone levels could potentially be the reason for a lack of current hydrocortisone thresholds in equine plasma.

Cortisone (Refer to figure 1.3 for the structure) is a naturally occurring glucocorticoid whilst synthetic cortisone was the first pharmacologically effective glucocorticoid to become readily available[81]. Cortisone converts from cortisol using the mineralocorticoid receptor and the glucocorticoid receptor as cortisol binds to these two receptors whilst cortisone does not[81]. The anti-inflammatory properties of cortisone have been extremely beneficial in both human and veterinary medicine[82]. Cortisone is generally used to treat muscular skeletal disorders in the horse however, they have been known to greatly affect a horse's mood and behaviour[82]. Synthetic version of cortisone must be enzymatically reduced before they are biologically

active to allow for absorption into the body[81]. With that however, the use of exogenous cortisone isn't as common compared to exogenous hydrocortisone. There is currently no international threshold for cortisone in either equine urine or blood.

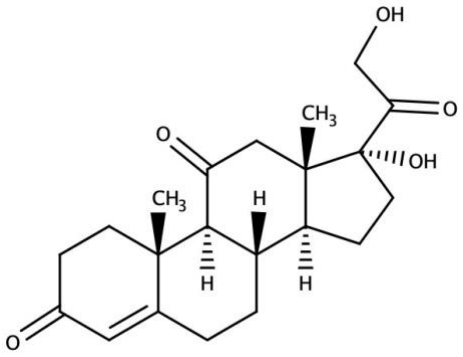

Figure 1.3: Structure of cortisone

In the biosynthesis pathway for steroids, hydrocortisone and cortisone are terminal compounds generated by the CYP11B1 (11-β-hydroxylase) enzyme (refer to figure 1.4 for the biosynthesis pathway for steroids). There are additional corticosteroids also formed using the CYP11B2 (18-hydroxylase) enzyme namely the terminal corticosteroids of 18-hydroxycortisol and 18-oxocortisol. Unfortunately, not much is known about these two terminal corticosteroids in the equine system however, given the location of these in the cascade, it would be beneficial to monitor these biomarkers for the possibility of detecting the effects of pharmaceuticals. Given the lack of knowledge of these two corticosteroids, there is also no current international threshold for these two compounds in equine urine or plasma.

Additionally, the biomarker of 11-deoxycortisol has been previously identified and quantitated in equine plasma, blood and serum[83-85]. Given its position in the pathway as the precursor to hydrocortisone, it could be extremely beneficial to monitor 11-deoxycortisol. It was concluded by Kirchmeier *et al.* that as 11-deoxycortisol increased 5.28-fold under the influence of an adrenocorticotropic hormone (ACTH) stimulation test, it would be beneficial to monitor 11-deoxycortisol to assess for stress in the horse[83]. From the study conducted by Genangeli *et al.* a method targeting 11-deoxycortisol (in addition to 17 steroidal homrones) was developed for equine serum however, when detected in four equine serum samples, the concentrations of which were very low or not detectable[84]. Following this, Caprioli *et al.*

further developed a method detecting 17 endogenous and exogenous steroidal hormones in equine and bovine blood which included 11-deoxycortisol[85]. In this study, the population study of 33 horses also showed low or non-detectable levels of 11-deoxycortisol[85]. Therefore, from these studies, it would be beneficial to detect for 11-deoxycortisol for this project as well. However, given the small population study conducted by the authors, there is currently a lack of international threshold in both equine urine and plasma for 11-deoxycortisol.

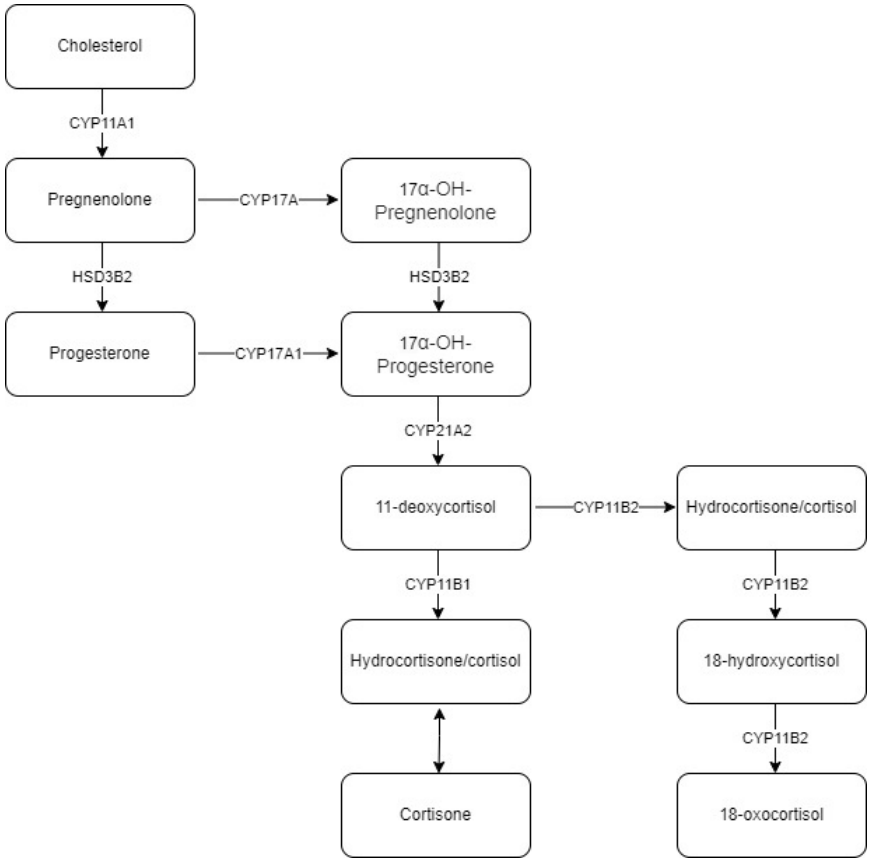

Figure 1.4: Biosynthesis pathway for corticosteroids starting from cholesterol. Figure adapted from a study by Mostaghel *et al.*, Kirchmeier *et al.*, and Lenders *et al.* [83,86,87]

1.10 Bisphosphonates and the lipidome

Bisphosphonates are a class of drugs first developed in the 1960s for the treatment of bone diseases related to bone resorption[88,89]. There are two classes of bisphosphonates, nitrogenous and non-nitrogenous with the introduction of a non-nitrogenous bisphosphonate (tiludronic acid) being first licenced in Europe in 2011 for veterinary medicine in the treatment of equine orthopaedic conditions[90,91]. Bisphosphonates are believed to have anti-

inflammatory and analgesic effects making them a potential for treatment in varying equine diseases[90]. It is hypothesised that the pain-relieving effects of bisphosphonates could be due to the reduction of inflammatory mediators such as PGE_2[90]. There is evidence that nitrogenous bisphosphonates have reduced serum concentrations of PGE_2, with equine studies showing pain relief following these administrations[90]. Unfortunately, the full mechanism of how bisphosphonates decrease pain in horses is unknown, but it is believed that pain-relieving mechanisms may be involved[88,90].

1.11 Aims of this Project

Currently in the field of equine anti-doping, there is limited literature of the monitoring for administrations using the lipid and corticosteroid biomarkers in equine plasma. There is also very little understanding of the basal levels of the lipid biomarkers and the effect of the exogenous corticosteroid and bisphosphonate administrations. The overarching aim for this project is to gain an understanding of the lipid and corticosteroid biomarkers with the hope it will allow for indirect detection for certain exogenous administrations.

The specific objectives of the project were:

- To pursue method validation for selected endogenous lipid and corticosteroid biomarkers in equine plasma. Method validation was completed for the biomarkers of oleoyl ethanolamide, arachidonoyl ethanolamide, hydrocortisone and cortisone.
- To complete a reference population of the selected biomarkers to determine basal levels with the absence of exogenous administrations. Samples were analysed from one specific location in New South Wales, Australia to allow for a general understanding of the levels present (n > 750 samples). The use of the reference population was to also identify biomarkers as an endogenous reference compound for the possibility of introducing a biomarker ratio.
- Completing administration studies focusing on four separate exogenous drugs, two corticosteroids and two bisphosphonates. This includes triamcinolone acetonide (TACA), fluticasone propionate (FLP), tiludronic acid (TA) and zoledronic acid (ZA). These four administrations will allow for the investigation on how endogenous lipid and corticosteroids change over time.

- To complete a translation of the selected lipids from a research perspective to routine analysis using an accredited routine method to optimise for high-throughput use.
- To monitor intra-individual profiles using selected biomarkers for horses on the equine biological passport.

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Chapter 2:
Endogenous Lipids and Corticosteroids

2.1 Introduction

Levels of endogenous lipids and corticosteroids are influenced by many factors including therapeutic treatment of medical conditions. These include exogenous administrations of corticosteroids (e.g. triamcinolone acetonide or fluticasone propionate) and bisphosphonates (e.g. tiludronic acid or zoledronic acid). The potential of utilising these lipids and corticosteroids as biomarkers to detect substance misuse in horse racing has not been explored. In 2009, the World Anti-Doping Agency (WADA), published the Athlete Biological Passport (ABP) to monitor specific biomarkers which may be affected for a longer duration than the original substance itself [92]. This idea combines longitudinal analysis of biomarkers and various other information that may be relevant, which may be helpful for targeted traditional anti-doping analysis [92]. Human anti-doping, however, has a very top-down approach where WADA will indicate the necessary biomarkers and substances each laboratory must detect for. Comparatively for equine anti-doping, there is a bottom-up approach where individual laboratories can indicate their testing scope and capabilities[71]. Using the idea of longitudinal profiling, Racing NSW has developed a similar database called the EBP.

The EBP attempts to detect substance misuse indirectly by monitoring profile change of biomarkers in response to the pharmacological effects of prohibited substances[71]. The urinary hydrocortisone internationally accepted threshold is 1 µg/mL however, there is a lack of an internationally accepted threshold in equine plasma[93]. The establishment of the EBP allows for the selection of substances that are considered important but do not have an accepted international threshold [71]. The EBP follows Bayesian modelling similar to those used in the ABP as presented by *Sottas et. al.* with the testosterone/epitestosterone ratio [94]. The EBP is an intelligence model that doesn't primarily rely on just analytical results collected from the laboratory [71]. It also includes data from stewards, animal welfare (the administration of therapeutic medications for treatment or the presence of underlying unknown health issues) and principal racing authorities for suspicious biological profiles of horses [71]. The EBP also provides a deterrent from the use of prohibited substances, as the EBP may show that the biological effect of the substance can be detected beyond the presence of the substance itself [71].

There are analytical challenges that need to be addressed for the use of endogenous biomarkers. A major issue with the quantification of endogenous compounds is the potential for interferences from the compound itself being present originally in the matrix [16]. Therefore, the use of a surrogate matrix is required. The use of surrogate matrices has been explored by *Thakare et al.* where various approaches were tested to determine the blank matrix that would be appropriate for accurate quantification [95]. Their study concluded that background subtraction and standard addition would be appropriate for methods that have endogenous levels of compounds affecting accurate quantification[95]. Standard addition however has the disadvantage of requiring large sample volumes to ensure an accurate quantification [95]. Other surrogates tested included neat solutions, artificial matrices, or stripped matrices where the endogenous compound is removed using charcoal cartridges (utilising SPE) or liquid-liquid extractions, but the remainder of the matrix is still present [15,95].

The second analytical challenge is the amount of volume used for the proposed method in monitoring the lipids and corticosteroids in equine plasma. Sample volumes can be limited after routine analysis due to the number of testing requirements necessary therefore, sample volumes had to be decreased to allow for additional testing methods. A smaller volume of sample can also encounter increase sensitivity issues on the instruments. Literature recommends the use of 200 µL of plasma due to ease of use, increased sensitivity and possibility of repeated samples if routine analysis showed abnormalities. The sample volume chosen for this project is 100 µL for harmonisation between the research methods for equine plasma at the Australian Racing Forensic laboratory. It is hypothesised that if the detection of lipids and corticosteroids is possible with 100 µL of equine plasma, the presence or absence would allow for direct or extended indirect detection for exogenous drug administrations. If successful, this method would also allow for the scope of biomarkers currently being monitored in routine practices to expand to include additional markers which are currently not being monitored nor have proposed international population reference limits.

These challenges could be overcome by developing a LC-HRMS method for the analysis of endogenous lipids and corticosteroids using 100 µL of horse plasma based on surrogate matrix calibrations. The LC-HRMS method used was previously developed by Shimadzu for the triple quadrupole mass spectrometer with this project translating that work onto the LC-HRMS QTOF. The 19 lipids present on the arachidonic acid cascade were selected to monitor

throughout this project include 11-Dehydro Thromboxane B_2 (11-dehydro TxB_2), 12-hydroxyeicosatetraenoic acid (12(S)-HETE), 13-hydroxydocosahexaenoic acid (13-HDHA), 15-hydroxyeicosadienoic acid (15-HEDE), 15(S)-hydroxyeicosatetraenoic acid (15(S)-HETE), 17-hydroxydocosahexaenoic acid (17-HDHA), 18-hydroxyeicosapentaenoic acid (18-HEPE), 5(S)-hydroxyeicosatetraenoic acid (5(S)-HETE), 6-Keto Prostaglandin $F1_\alpha$ (6-Keto $PGF_{1\alpha}$) , 9-hydroxyoctadecatrienoic acid (9-HOTrE), Arachidonic Acid (AA), Arachidonoyl Ethanolamide (AEA), Leukotriene B_4 (LTB_4), Leukotriene D_4 (LTD_4), Leukotriene E_4 (LTE_4), Oleoyl Ethanolamide (OEA), Prostaglandin D_2 (PGD_2), Prostaglandin E_2 (PGE_2), Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). These lipids have all be identified as lipids of inflammation which would be beneficial with the aim of exploring if these biomarkers are affected by other drug administrations. Refer back to chapter 1.3 for the description of each lipid and its position on the AA cascade. Selected corticosteroids were also chosen to investigate baseline levels as the arachidonic acid cascade focuses on inflammation, it was hypothesised endogenous corticosteroids could be affected as well. With that, 5 endogenous corticosteroids including hydrocortisone (HC), cortisone (C), 18-oxocortisol, 18-hydroxycortisol and 11-deoxycortisol were additionally monitored. Due to the terminal positions on the biosynthesis pathway, these corticosteroids were monitored under the influence of varying drug administrations to determine their effect (refer to chapter 1.9 for description of each corticosteroid). Additional data processing involving semi-targeted (semi-quantitative using transitions already present in the method package) and untargeted analysis were also completed.

2.1.1 Aims

This chapter explores the 19 lipid and 5 endogenous corticosteroid biomarkers using 100 µL of equine plasma. A 100 µL method was developed in reference to the original Shimadzu method package for detection of lipids on the triple quadrupole mass spectrometer[16,96]. As the original method package utilised only 30 µL of human plasma and with the knowledge that the LC-HRMS used for this project is less sensitive than the triple quadrupole, the volume of sample was increased to 100 µL of equine plasma. The use of 100 µL of equine plasma for this project was primarily due to limited sample availability but also allowed for harmonisation for this assay in the laboratory to previously developed metabolomics methods by Keen *et al.*[97]. The method was applied to investigate the basal levels of the lipid

and corticosteroid analytes in horse plasma from population samples and EBP samples to derive population reference limits (PRL) and individual reference limits (IRL), respectively, for antidoping applications. This is due to the lack of understanding of these biomarkers and the overall concentrations of these biomarkers within the horse. With this understanding, the equine anti-doping community will have the ability to extend the scope of the detection of administrations using indirect detection further complementing current detection methods. Numerous studies have been completed on varying drug administrations to monitor how the lipid biomarkers are affected as reviewed by Tou *et al*[98]. and seen in chapter 1. This study however would be the first to monitor the arachidonic acid cascade and the corticosteroid biosynthetic pathway in a holistic view as there is currently a lack of literature detailing the concentrations of these lipids for antidoping purposes in horse racing.

2.2 Materials and Methods

2.2.1 Chemicals and Reagents

LC grade dichloromethane (DCM), hexane and isopropanol (IPA) were purchased from Merck (Castle Hill, NSW, Australia). Acetonitrile (ACN), formic acid (FA) and methanol (MeOH) of MS grade and ethanol (EtOH) of LC grade were purchased from ThermoFisher (Waltham, Massachusetts, USA). Water used was ultrapure grade (18.2 MΩ cm) obtained from a ThermoFisher Barnstead Smart2Pure system (Langenselbold, Hungary).

2.2.2 Reference Materials

Reference material of the analytes 11-dehydro thromboxane B_2, 12(S)-HETE, 13-HDHA, 15-HEDE, 15(S)-HETE, 17-HDHA, 18-HEPE, 5(S)-HETE, 6-Keto Prostaglandin $F1_\alpha$, 9-HOTrE, Arachidonic Acid, Arachidonoyl Ethanolamide, Leukotriene B_4, Leukotriene D_4, Leukotriene E_4, Oleoyl Ethanolamide, Prostaglandin D_2, Prostaglandin E_2, Prostaglandin $F_{2\alpha}$ were manufactured by Cayman Chemicals (Ann Arbor, Michigan USA) and purchased from Sapphire BioScience (Redfern, NSW, Australia).

The corresponding internal standards of Thromboxane B_2-D_4, 11(12)-EET-D_{11}, 12(S)-HETE-D_8, 14(15)-DiHET-D_{11}, 15-HETE-D_8, 5-HETE-D_8, 6-Keto Prostaglandin $F_{1\alpha}$, Arachidonic Acid-D_8,

Leukotriene B_4-D_4, Leukotriene D_4-D_5, Oleoyl Ethanolamide-D_4, Prostaglandin D_2-D_4, Prostaglandin E_2-D_4 and Prostaglandin $F_{2\alpha}$-D_4 were manufactured by Cayman Chemicals (Ann Arbor, Michigan USA) and purchased from Sapphire BioScience (Redfern, NSW, Australia).

Hydrocortisone (HC) and cortisone (C) were obtained from Merck (Castle Hill, NSW, Australia). 18-oxocortisol was manufactured from Toronto Research Chemicals (Toronto, Ontario, Canada), 18-hydroxycortisol and 11-deoxycortisol were manufactured from IsoSciences (Ambler, Pennsylvania, USA) and purchased from PM Separations (Capalaba, QLD, Australia). The internal standard of Hydrocortisone-D_4 was obtained from Cambridge Isotope Laboratories (Andover, Massachusetts, USA).

2.2.3 Surrogate Matrix

A surrogate matrix was utilised due to the endogenous nature of the target compounds. Blank plasma pooled from a collection of equine plasma known to not contain any exogenous drugs was used to produce the surrogate matrix. Blank plasma was transferred into screw top test tubes. Liquid-liquid extraction was performed using 3 mL plasma aliquots and 4 mL of a solution of DCM and EtOH in a 90:10 ratio. Solutions were rotated for 20 minutes at medium speed (15 rpm) allowing for mixing between the plasma and extraction solution (DCM : EtOH (90:10 v/v). Each tube was then centrifuged at 3000 rpm for 10 minutes. Following centrifugation, the aqueous plasma layer from each tube was transferred into glass tubes and stored at -20 °C until use was required.

2.2.4 Blood Samples – Routine Race Day and EBP Sampling

Reference population samples were compiled using race-day samples from September 2021 to May 2023. These samples were collected from pre-race samples at the Australian Turf Club in New South Wales, Australia. Blood samples were also collected from passport horses that participate in races at the Australian Turf Club (horses 1 (male), 2 (female) and 3 (gelding)). All blood samples were collected in BD Lithium Heparin Vacutainer® (Mississauga, ON, Canada) tubes (Cat # 367880) by veterinarians and swab officials employed by Racing NSW. Samples were transported chilled to the Australian Racing Forensic Laboratory (ARFL) where,

on arrival and after routine checking to ensure forensic integrity, were stored at 4 °C until analysis which was usually within 7 days of arrival at the laboratory.

The use of race-day samples for reference population studies in addition to longitudinal profiling of passport horses was approved by the Racing NSW Animal Care and Ethic Committee (ARA 80).

2.2.5 Lipid Extraction Method

The method was adapted from Toewe *et al.* for comprehensive analysis of lipid mediators, in human plasma[16]. An aliquot of 100 µL of plasma was transferred into a 1.5 mL microcentrifuge tube (Reaktionsgeäße 3810X, Eppendorf, Hamburg, Germany). Protein precipitation was completed using 300 µL of 0.1% FA in MeOH. A mixed working solution containing the 14 lipid internal standards was made to a concentration of 60 ng/mL (6-K $PGF_{1\alpha}$, TxB_2-D_4, $PGF_{2\alpha}$-D_4, PGE_2-D_4, PGD_2-D_4, LTD_4-D_5, LTB_4-D_4, 14,15-DiHET-D_{11}, 15-HETE-D_8, 12-HETE-D_8, 5-HETE-D_8, PAF C-16-D_4 and 11,12-EET-D_{11}), 12 ng/mL (OEA-D_4), 1200 ng/mL (AA-D_8) and 50 ng/mL (HC-D_4). 10 µL of this mixed internal standard solution was spiked into each sample before rotary mixing for 3 minutes at 4 °C followed by centrifugation using a Beckman Coulter Microfuge 20 at 13000 rpm for 10 minutes. The supernatant was transferred into a glass test tube with 900 µL of 0.1% FA in H_2O added and then lightly agitated before solid phase extraction (SPE).

SPE was completed on a UCT positive pressure manifold using Phenomenex Strata-X 10 mg reversed phase cartridge (Torrance, California, USA). The cartridge was conditioned with 1 mL of 0.1% FA in MeOH and 0.1% FA in H_2O. Samples were loaded before being washed with 1 mL of 0.1% FA in H_2O, 0.1% FA in 15% EtOH and hexane. The target fraction was then eluted using 300 µL of 0.1% FA in MeOH before being dried using a Genevac EZ-2 evaporator at 45 °C for 90 minutes. Samples were reconstituted in 100 µL of 0.1% FA in MeOH in an autosampler vial and stored at -20 °C until LC-HRMS analysis.

2.2.6 Instrument Parameters

LC-HRMS analysis was performed using a LC40 liquid chromatograph (LC) coupled to a 9030 quadrupole-time of flight (QTOF) mass spectrometer from Shimadzu Scientific Instruments

(Kyoto, Japan). Separation was performed using a Phenomenex Kinetex C8 column (2.1 mm x 150 mm, 2.6 µm) (Torrance, California, USA) using a gradient elution. Aqueous mobile phase A consisted of 0.1% FA in H_2O whilst organic mobile phase B was ACN. The gradient was: 0 min A-B (90:10 % v/v), 0-5 minutes A-B (75:25 % v/v), 5-10 minutes A-B (65:35 % v/v), 10-20 minutes A-B (25:75 % v/v), 20-28 minutes A-B (2:98 % v/v) and 28-30 minutes (90:10 % v/v) as post equilibration time. The flow rate was constant at 0.4 mL/min with an injection volume of 5 µL and the column temperature was set to 40 °C.

Shimadzu LabSolutions software (Version: 5.99 SP2) was used for data acquisition. MS data was collected between 1 to 25 minutes Data was collected using data independent acquisition (DIA) mode in both ESI+ and ESI- with the *m/z* range between 50-700 at a resolution of 30,000 full width half maximum (FWHM). The MS conditions are as stated in appendices Table A1.

2.2.6.1 Data Acquisition Experimentation

Three different types of data acquisition were experimented to determine which method would be most appropriate for the capabilities of this method. The three types of acquisition which were tested included data independent acquisition (DIA), data dependent acquisition (DDA) and a targeted MS2 method. Table 2.1 highlights the varying conditions for each to determine which acquisition would be appropriate.

Table 2-1: Data acquisition experimentation conditions

Conditions	**Data independent acquisition (DIA)**	**Data dependent acquisition (DDA)**	**Targeted MS2**
Precursors	300 – 600 m/z (in 10 m/z windows)	100 -700 m/z	Optimised pre-cursor ions
Collision energy (CE)	3.0 – 31.0 eV (positive) 1.0 – 35.0 eV (negative)	18.0 – 52.0 eV	Optimised collision energy spreads for each specific compound
Event Time	MS1 = 0.100 s MS2 = 0.030 s	0.100 s	MS1 = 0.500 s MS2 = 0.040 s

MS2 range:	50 – 700 m/z	50 – 700 m/z	Product ion scans and multiple reaction monitoring transitions optimised for each ion
Other specifications		Exclusion list based off a sample of phosphate buffer saline (PBS)	

2.2.7 Method Validation Preparation

The method was validated for the quantification of HC, C, OEA and AEA. The parameters assessed were linearity, sensitivity, accuracy, precision, recovery, matrix effects and stability as outlined by Peters *et al*[99]. These method validation parameters will be referenced in other experimental chapters as necessary.

2.2.7.1 Linearity

A calibration curve was used to establish the linearity of the response ratio of target compounds using their relevant internal standard in relation to concentration. The R^2 value is indicative of how linear the curve is with values as close to 0.999 being ideal. Linearity was assessed for calibration concentrations of 0, 1, 2, 5, 10, 50, 100 and 200 ng/mL for OEA and AEA as spikes in the plasma matrix. Individual 2000 ng/mL working solutions were made for the 4 compounds from solutions of 1000 µg/mL for OEA, 50,000 ug/mL for AEA, 1019 µg/mL for HC and 1034.1 ug/mL for C to allow for spiking into surrogate matrix equine blank plasma. From the 2000 ng/mL working solution, a 10 ng/mL and 200 ng/mL solution was made for the plasma spike. The spike amounts were determined using equation 2.1. The amounts used for each concentration can be visualised in Table A2 in the appendices. Plasma spikes were replicated four times to account for any variabilities which may be associated with either the sample preparation or instrument.

$$Spike = \frac{Final\ Concentration\ needed \times Total\ amount\ of\ plasma}{Original\ Concentration}$$ (Equation 2.1)

The accuracy of the regression analysis was assessed by analysing the residuals from the linearity curve for each compound. This was done by analysing each replicate of the linearity curve and using the equation 2.2 to calculate the residual with the expected concentration determined from the linearity curve and the calculated concentration determined by the LabSolutions Insight software.

$$Residual = Expected\ Concentration - Calculated\ Concentration$$ (Equation: 2.2)

2.2.7.2 Sensitivity

Sensitivity was tested by determining the limit of detection (LOD) and limit of quantification (LOQ) by visual comparison of the signal-to-noise ratio (*S/N*) of 3 for the LOD and 10 for the LOQ in accordance with the Association of Official Racing Chemists guidelines[100,101]. Concentrations of 0.4, 0.3, 0.2 and 0.1 ng/mL were chosen for this assessment with Table A3 in the appendices providing the total amounts required from a 1 ng/mL working solution.

2.2.7.3 Accuracy

Accuracy was assessed using freshly prepared replicates of 7 plasma spikes. For the method to be deemed accurate, the percentage relative error (% RE) needed to be $\leq$ 15%[99]. Accuracy was estimated by calculating the relative error from the average concentration of the 7 replicates compared to the theoretical concentration using the equation 2.3.

$$Relative\ Error = \left(\frac{(Calculated\ Concentration - Theoretical\ Concentration)}{Theoretical\ Concentration}\right) 100$$ (Equation 2.3)

2.2.7.4 Precision

Precision was assessed using the averaged concentrations of the 7 replicates with the percentage relative standard deviation (% RSD) being $\leq$ 15%[99]. The RSD was calculated using equation 2.4 with standard deviation and averages being calculated using the respective *Excel* functions.

$\%\ RSD = \left(\frac{Standard\ Deviation}{Mean\ Concentration}\right) \times\ 100$ (Equation 2.4)

2.2.7.5 Recovery

Recovery was assessed using 7 equine plasma spike replicates with concentrations spiked either at pre- or post-extraction at the same concentrations as the other method validation requirements using equation 2.5. Recovery was expected to be higher than 50%[99] which is considered to be an acceptable range given the endogenous nature of the compounds. The average recovery was determined using the average function in *Excel*.

$Recovery = (\frac{Average\ concentration\ of\ pre\ extraction\ spikes}{Average\ concetration\ of\ post\ extraction\ spikes})\ x\ 100$ (Equation 2.5)

2.2.7.6 Matrix Effects

Matrix effects (ME) were assessed by comparing the response of 7 replicate post-extraction spiked matrix samples to 7 neat standards. ME were essential to consider for the LC-MS method utilising electrospray ionisation mode. This form of method validation gives the information as to whether the analytes underwent ion enhancement or suppression during instrument analysis. If the percentage of the ME was greater than 100%, the analyte was deemed to have undergone ion enhancement whilst a percentage less than 100% would be concluded as having undergone ion suppression[102]. The ME was estimated using equation 2.6 with the average ME being determined using the average function in *Excel*.

$ME = \left(\frac{Average\ area\ of\ post\ extraction\ spikes}{Average\ area\ of\ neat\ standards}\right) x\ 100$ (Equation 2.6).

2.2.7.7 Stability

Stability was analysed over a 4-week period with samples analysed at the 2-weeks, 3-weeks, and 4-weeks in duplicate. Stability was assessed at the same concentrations as the previous method validation requirements and were stored at either 4 °C or – 20 °C. At the allocated time point, the samples were thawed to room temperature and prepared using the method as outlined in chapter 2.2.5. Concentrations were calculated utilising the *LabSolutions Insight* software. The average concentration for each temperature condition calculated for the

associated time period using the average function in *Excel*. Any samples outside of a 20% margin were considered unstable[99].

2.2.8 Data Analysis and Processing Parameters

All data was processed using the Shimadzu *Insight Explore* software (Version: 3.8 SP1) with further data processing completed with *Excel* (version 16.71), MATLAB (Version R2021B) or *MetaboAnalyst* (Version 5.0).

2.2.8.1 Raw Data and Targeted Screening

Concentrations were determined using linear regression from the calibration curve being the area ratio of the target compound to relevant internal standard response. Table 2.2 shows the *LabSolutions Insight* conditions for quantification of the compounds (applicable for positive and negative).

For administration samples, percentage change, fold change and p-values were calculated for relevant compounds to determine significance. As outlined by Yu *et. al* where a fold change (FC) of ≥ 1.5 for up-regulation or ≤ 0.67 for down-regulation in addition to a p-value of less than 0.05 being deemed a significant value in comparison to pre-administration samples[103].

Table 2-2: Parameters for LabSolutions Insight Explore acquisition data for LC-HRMS 9030

Condition	Optimised Condition
Peak Integration Conditions	
Smoothing Method	Standard
Smoothing Width	3 seconds
Identification Conditions	
Identification Method	Absolute
Peak Selection	Closest Peak
Window for Target Peak	5.00%
Window for Reference Peak	5.00%
Processing Time	± 1.5 min

Quantitative Conditions	
Method	Internal Standard
Calculated by	Area
Number of calibration levels	8
Type	Linear
Zero	Not Forced
Units	ng/mL

2.2.8.2 Semi-Targeted Screening

Semi-targeted analysis of lipids was performed using the Shimadzu lipid exact mass method package containing 196 lipids and 18 internal standards with retention time matching for isomer assignment acquired in both positive and negative ESI modes. Criteria was set for confirmation of analytes with chromatography peaks having a signal to noise ratio of three or more, retention time within 0.1 minutes of that expected by the method package, the acquired mass of the compound being within 5 ppm error of the theoretical mass, the respective area of the peak is to be greater than 500 counts and the peak is of good shape with a flat baseline requiring little to no extra integrating. Compounds that fit this set criteria would have the peak area plotted in Excel to determine if there was distinct activity for further analysis.

2.2.8.3 Non-Targeted Screening

DIA enabled untargeted analysis to be investigated for at selected time points for the 2 corticosteroid and 2 bisphosphonate administrations which will be discussed in later chapters. These were chosen with respect to maxima in the elimination curves for detected compounds. A workflow was developed using the Shimadzu *Insight Explore* software utilising the naïve feature finding algorithm. This algorithm provides a list of pre-cursor ions with high abundance and generates a list that contains the retention time, mass, and peak area. This data was transferred to *MetaboAnalyst* for statistical analysis. Statistical tests including PCA, volcano plots and heat maps were utilised to determine up- and down-regulated compounds.

Selected compounds were transferred back into *Insight Explore* and compared with the initial generated compound list to determine the mass of the compound. As *Insight Explore* also has the additional function of being able to predict the formula, compounds with mass error formulas less than 5 ppm error were also noted.

2.2.9 Reference Population Data Analysis

Data analysis for the reference population was completed using *Microsoft Excel for Mac* (version 16.76) using basic statistical functions including mean, standard deviation, frequency, kurtosis, skewness, and quartile analysis. Box and whisker plots for gender comparisons and normality population plots were generated using *MATLAB* (version R2023b) with functions from the additional Statistics and Machine Learning Toolbox (version 23.2).

Testing for normality was completed by transforming the data using a log normal transformation, probability plots were then produced in *MATLAB* (version R2023b) to determine the parametric range. Once a data set is determined to follow a parametric distribution, the threshold equation could then be used to propose an upper or lower threshold for the relative compound. Equation 2.7 shows the equation used for this proposal. This equation is commonly used by racing authorities to propose endogenous thresholds with the use of the 3.72 allowing for 99.99% of the confidence level considered in accordance to the guidelines as set out by the Association of Official Racing Chemists[104]. The sum or difference in the threshold equation allows for the result from the log transformed concentration, this number is then untransformed using the exponential function in *Excel* to obtain either an upper or lower threshold.

$$Threshold = Mean \pm (Standard\ deivation \times 3.72)$$ (Equation 2.7)

To propose a threshold for non-parametric distribution, the data was firstly sorted into ascending order and the 0.005th and 99.995th percentile was determine as the lower and upper threshold respectively[105].

2.2.10 Equine Biological Passport – Individual Reference Limits

For data obtained for the EBP, profiles are modelled utilising a formula derived by McIntosh *et al.* based on the parametric empirical Bayes method[106,107]. This formula utilises either the sum or difference allowing for an individual's upper or lower reference limit (IRL) to be derived. Equation 2.8 and 2.9 show the formula to derive the IRLs with equation 2.10 indicating how the intra-class correlator (B_n) is calculated.

$$Upper\ Reference\ Limit\ =\ \mu + (\bar{x} - \mu)\ B_n + \ Z_\alpha \sqrt{1 - B_1 B_n}\ \sqrt{V}$$ (Equation 2.8)

$$Lower\ \ Reference\ Limit\ =\ \mu + (\bar{x} - \mu) B_n - \ Z_\alpha \sqrt{1 - B_1 B_n}\ \sqrt{V}$$ (Equation 2.9)

$$B_n = \frac{\sigma^2}{s^2/n + \sigma^2}$$ (Equation 2.10)

$$B_1 = \frac{\sigma^2}{V}$$ (Equation 2.11)

Within each equation, μ is the population mean, $\bar{x}$ refers to the individual mean, Bn is the intra-class correlator, Zα refers to the confidence level used for each individual profiles, B_1 is the intra-class correlator of 1 for an individual profile and V refers to the total variance. For the intra-class correlator equation (equation 2.10), σ^2 refers to the log transformed population mean squared, s^2 refers to the standard deviation of the log transformed values squared and n refers to the number of samples for each individual horse. Bn refers to the intra-class correlator for the number of samples for the individual horse whilst the B_1 term indicates the intra-class correlator for the first sample (equation 2.11). The intra-class correlator is one of the most important terms in both of these equations this term is heavily reliant on the number of samples each individual horse has. As more samples are taken from the horse and incorporated into the profile, the weight of the individual variance increases as the population variance decreases. For individual profiles, a confidence level of 3.09 is used which relates to a 99.9% confidence level. This is in comparison to any reference population statistics which rely on a confidence level of 3.72 equating to a 99.99% confidence level.

2.3 Results and Discussion

2.3.1 Optimised LC Conditions

Retention times for each compound were checked using the highest calibrator for each sequence to ensure compliance with Association of Official Racing Chemists (AORC) criteria[101]. Each retention time was expected to fall within ± 0.2 minutes of the corresponding reference material [101]. The retention times for the lipids and internal standard is presented in Table 2.3 whilst for corticosteroids and internal standards is presented in Table 2.4. For 15-HEDE, the original internal standard of 11(12)-EET-D_{11} was not detectable. Therefore, the internal standard of 14,15-DiHET-D_{11} was chosen as the internal standard for 15-HEDE. Similarly for 11-deoxycortisol, there was no available internal standard at time of method optimisation, therefore the hydrocortisone-D_4 was selected as the internal standard. To calculate the relative retention time, equation 2.13 was used.

$$Relative\ Retention\ Time = \frac{Retention\ Time\ of\ Compound}{Retention\ Time\ of\ Internal\ Standard\ Used} \quad \text{(Equation: 2.13)}$$

Table 2-3: Retention time and relative retention time for lipids under the optimised LC conditions

Compound	Retention Time (min)	Internal Standard Used	Relative Retention Time
6-Keto Prostaglandin $F_{1\alpha}$	7.68	6-Keto Prostaglandin $F_{1\alpha}$-D_4	1.01
Thromboxane B_2	9.46	Thromboxane B_2-D_4	1.00
Prostaglandin $F_{2\alpha}$	10.27	Prostaglandin $F_{2\alpha}$-D_4	1.00
Prostaglandin E_2	10.70	Prostaglandin E_2-D_4	1.00
11-Dehydro Thromboxane B_2	10.77	Prostaglandin E_2-D_4	1.01
Prostaglandin D_2	11.10	Prostaglandin D_2-D_4	1.00
Leukotriene D_4	12.67	Leukotriene D_4-D_5	1.00
Leukotriene E_4	12.86	Leukotriene D_4-D_5	1.02

Leukotriene B_4	14.12	Leukotriene B_4-D_4	1.00
9-HOTrE	15.68	14,15-DiHET-D_{11}	1.05
18-HEPE	15.86	14,15-DiHET-D_{11}	1.06
15(S)-HETE	16.93	15(S)-HETE-D_8	1.00
17-HDHA	17.08	12-HETE-D_8	0.99
13-HDHA	17.15	12-HETE-D_8	0.99
12(S)-HETE	17.26	5(S)-HETE-D_8	0.99
5(S)-HETE	17.36	5(S)-HETE-D_8	1.00
15-HEDE	18.15	14,15-DiHET-D_{11}	1.21
AEA	18.21	OEA-D_4	0.95
OEA	19.14	OEA-D_4	1.00
Arachidonic Acid	20.32	Arachidonic Acid – D_8	1.00

Table 2-4: Optimised LC conditions for endogenous corticosteroids

Compound	**Retention Time (min)**	**Internal Standard Used**	**Relative Retention Time**
Hydrocortisone	7.33	Hydrocortisone-D_4	1.01
Cortisone	7.48	Hydrocortisone-D_4	1.03
18-Oxocortisol	5.28	Hydrocortisone-D_4	0.73
18-Hydroxycortisol	5.30	Hydrocortisone-D_4	0.73
11-Deoxycortisol	9.78	Hydrocortisone-D_4	1.35

2.3.2 Optimised MS Conditions

The optimisation of MS conditions was to ensure the correct compound separation based off of the relevant accurate mass to 4 decimal places. Table 2.5 represents the acquisition mode and accurate mass as acquired from the instrument for lipids and Table 2.6 represents the same information for corticosteroids.

Table 2-5: Accurate masses for lipids under optimised MS conditions

Compound	**Acquisition Mode**	**Accurate Mass (*m/z*)**	**Protonated/Deprotonated Mass (*m/z*)**
6-Keto Prostaglandin $F_{1\alpha}$	Negative	370.4860	369.2283
Thromboxane B_2	Negative	370.4860	369.2283
Prostaglandin $F_{2\alpha}$	Negative	354.4870	353.2334
Prostaglandin E_2	Negative	352.4870	353.2334
11-Dehydro Thromboxane B_2	Negative	368.4700	367.2126
Prostaglandin D_2	Negative	352.4710	351.2177
Leukotriene D_4	Positive	496.6630	497.2680
Leukotriene E_4	Positive	439.6110	440.2465
Leukotriene B_4	Negative	336.4720	335.2228
9-HOTrE	Negative	294.2190	293.2122
18-HEPE	Negative	318.2190	317.2122
15(S)-HETE	Negative	320.4730	319.2279
17-HDHA	Negative	344.2346	343.2279
13-HDHA	Negative	344.2346	343.2279
12(S)-HETE	Negative	320.2346	319.2279
5(S)-HETE	Negative	320.4730	319.2279
15-HEDE	Negative	324.2659	323.2592
AEA	Positive	347.5430	348.2897
OEA	Positive	325.5370	326.3054
Arachidonic Acid	Negative	304.4740	303.2330
6-Keto Prostaglandin $F_{1\alpha}$-D_4	Negative	374.5104	373.2679
Thromboxane B_2-D_4	Negative	374.5104	373.2679
Prostaglandin $F_{2\alpha}$-D_4	Negative	358.5114	357.2585
Prostaglandin E_2-D_4	Negative	356.4954	355.2428

Prostaglandin D_2-D_4	Negative	356.4954	355.2428
Leukotriene D_4-D_5	Positive	501.6935	502.2994
Leukotriene B_4-D_4	Negative	340.4964	339.2479
14(15)-DiHET-D_{11}	Negative	349.3142	348.3075
15(S)-HETE-D_8	Negative	328.5218	327.2781
12(S)-HETE-D_8	Negative	328.2848	327.2781
5(S)-HETE-D_8	Negative	328.5218	327.2781
OEA-D_4	Positive	329.5614	330.3305
Arachidonic Acid-D_8	Positive	312.5228	311.2832

Table 2-6: Accurate masses for corticosteroids under optimised MS conditions

Compound	**Acquisition Mode**	**Accurate Mass (*m/z*)**	**Protonated Mass (*m/z*)**
Hydrocortisone	Positive	362.2093	363.2166
Cortisone	Positive	360.1937	361.2010
18-Oxocortisol	Positive	376.4490	377.1959
18-Hydroxycortisol	Positive	378.4650	379.2115
11-Deoxycortisol	Positive	346.4670	347.2217
Hydrocortisone-D_4	Positive	366.2344	367.2417

2.3.3 Acquisition methods

2.3.3.1 Data acquisition comparisons

Using the QTOF instrument, three acquisition modes, DIA DDA and the more conventional targeted MS2, were tested. An evaluation was performed to assess the strengths and weaknesses for each of these and implement the optimised analysis method. For the lipids, with the possibility of potential matrix effects each compound required individual assessment due to their unknown presence (some lipids may produce better sensitivity with DDA or some with DIA). Therefore, for this pilot experiment, it was ideal to choose two representative

analytes, HC and C given their known presence in equine plasma. The following product ions (as seen in Table 2.7) were monitored after a MS2 targeted method was completed for HC and C.

Table 2-7: Product ions to monitor for acquisition experiment.

Compounds	**Product Ions to Monitor (*m/z*)**
Hydrocortisone	121.0642 309.1849 327.1953 267.1742
Cortisone	163.1111 265.1589 343.1911 121.0641
Hydrocortisone-D_4	121.0644 331.2205 313.2097 271.1993

Comparisons were made between the three types of acquisitions with a focus on determining which method would be most appropriate for either a confirmation or screening purpose (Table 2.8).

Table 2-8: Targeted MS2, DDA and DIA for confirmations using HC only.

Conditions	**Targeted MS2 (refer to figure 2.1 for spectrum)**	**DDA (refer to figure 2.2 for spectrum)**	**DIA (refer to 2.3 for spectrum)**
Collision energy (CE) (eV)	19.0 – 23.0	18.0 – 52.0	3.0 – 31.0
Base peak (*m/z*: 121.06) height	2.35×10^4	1.00×10^4	7.92×10^3

Precursor (*m/z*): 363.21) height	2.04 x 10^4	2.83 x 10^3	3.80 x 10^4
Mass error	< 5 ppm	< 5 ppm	1 – 10 ppm
Other notable distinctions	Other product ions high in abundance	Product ions hidden amongst noise	Product ions very low in abundance

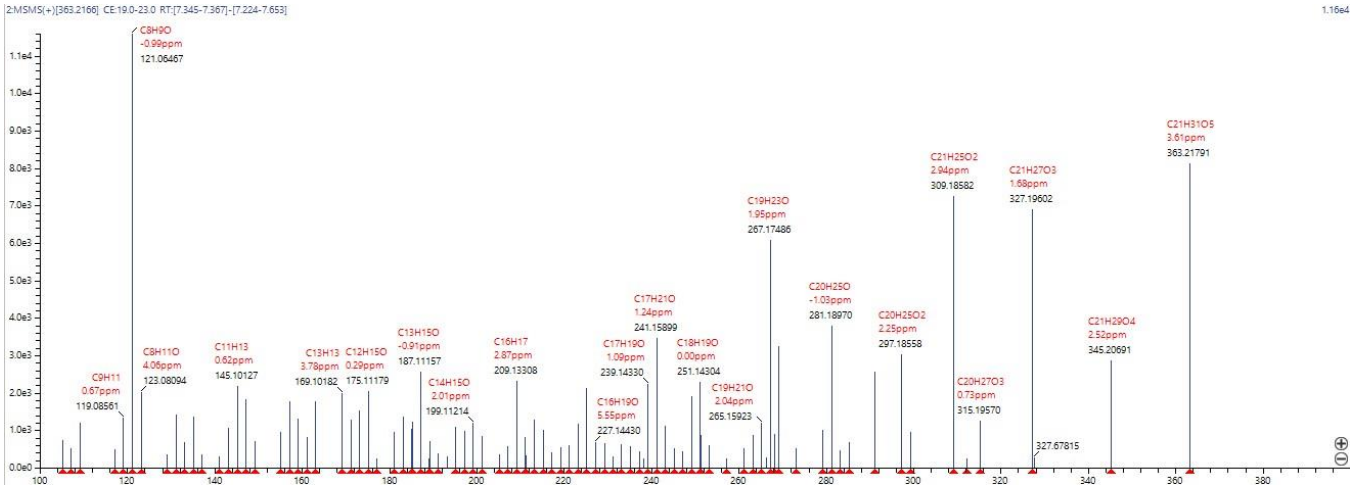

Figure 2.1: Targeted MS2 acquired data for HC.

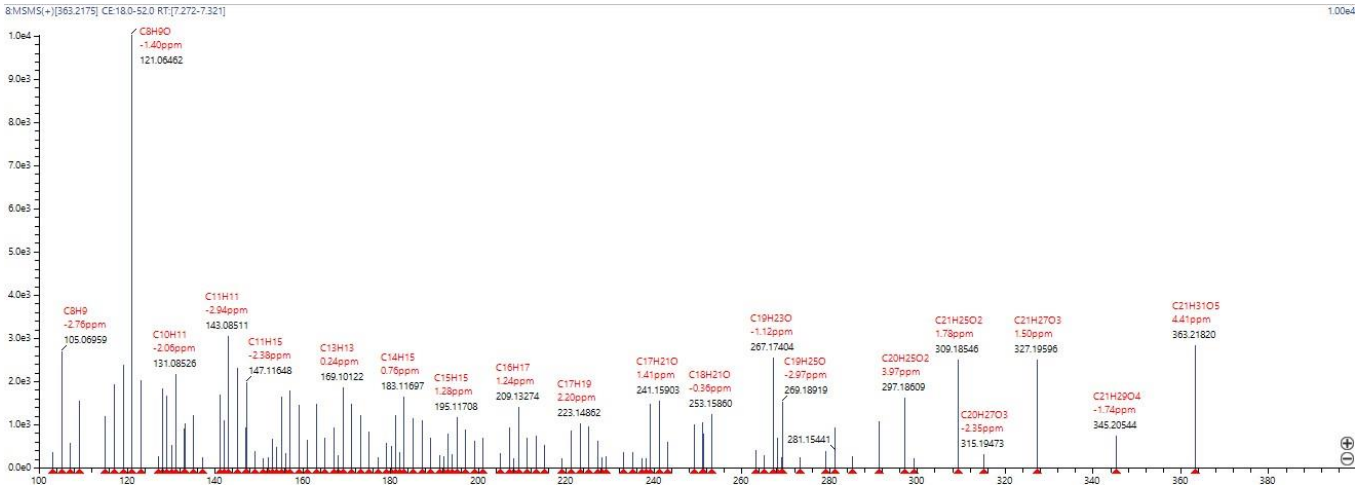

Figure 2.2: DDA data for HC.

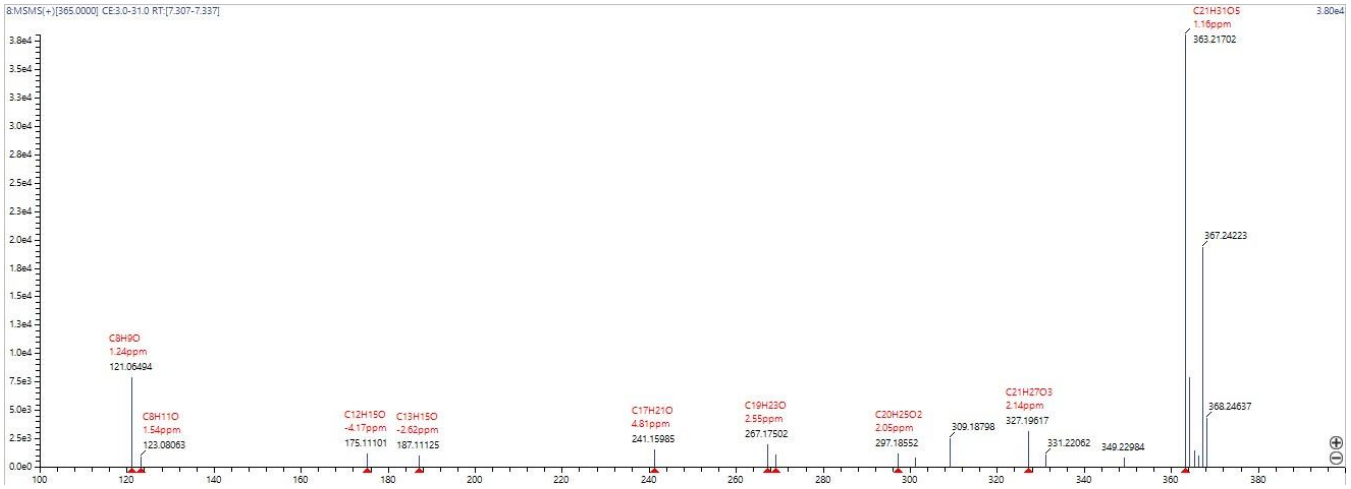

Figure 2.3: DIA data for HC.

Using HC as the example given its high abundance in equine plasma, the targeted MS2 optimised method provided product ions high in abundance with a large base peak ion. Comparatively for DDA, the product ions were not as characteristic and are hidden amongst noise notably due to the large collision energy spread required for this form of acquisition (Figure 2.2). From the DIA spectrum, the main difference is the collision energy spread is set to 3.0 – 31.0 which may account for the product ions not being in high abundance compared to the targeted MS2 method. Therefore, from the evaluation in this study, for confirmatory purposes, the targeted MS2 method would be most appropriate, however, either DDA or DIA may be used for the analysis of HC which was explored further.

Whilst the use of confirmatory testing is important to identify specific compounds present in the sample and/or endogenous up- or down-regulated biomarkers, screening must precede any confirmation. Both are complementary since there cannot be a confirmation without having an idea first of what could possibly be in a sample. With that in mind, the comparison between DDA and DIA for screening purposes was conducted. Table 2.9 provides the results for a DDA screening method using an exclusion list but no inclusion list. Table 2.10 shows DDA with both an inclusion and exclusion list and Table 2.11 provides the results for DIA for screening purposes.

Table 2-9: DDA with exclusion list used (no inclusion list) for screening purposes.

Compound and precursor mass (*m/z*)	S/N	Present in MS1	Fragmentation for M2?
Hydrocortisone (363.21688)	296	Yes	Yes
Cortisone (361.2010)	4	Yes	No
Hydrocortisone-D_4 (367.24201)	55	Yes	Yes

Table 2-10: DDA with exclusion and inclusion list for screening purposes.

Compound and precursor mass (*m/z*)	S/N	Present in MS1	Fragmentation for M2?
Hydrocortisone (363.21688)	241	Yes	Yes
Cortisone (361.2010)	5	Yes	No
Hydrocortisone-D_4 (367.24201)	53	Yes	Yes

Table 2-11: DIA for screening purposes

Compound and precursor mass (*m/z*)	S/N	Present in MS1	Fragmentation for M2?
Hydrocortisone (363.21688)	1183	Yes	Yes
Cortisone (361.2010)	15	Yes	Yes
Hydrocortisone-D_4 (367.24201)	425	Yes	Yes

Looking at Tables 2.9, 2.10 and 2.11, DDA with an exclusion list only and DDA with both an inclusion and exclusion list, had all 4 ions present in the MS1 spectra however, due to the low intensities of C, this did not produce an MS2 spectra. Therefore, with the use of DDA for screening purposes, there is the necessity to run samples using multiple methods at lower intensity thresholds to obtain the MS2 for compounds such as C. However, with the need to run at lower intensity thresholds, there is the possible risk of fragmenting noise rather than other compounds in the sample. Comparatively using DIA, all compounds were seen in MS1 with all being fragmented to produce MS2 spectra with good fragmentation patterns.

Therefore, for screening purposes, the use of DIA is preferred due to its ability to fragment compounds with lower intensities into MS2 with one run.

With this project requiring screening for targeted, semi-targeted and untargeted purposes, DIA was selected to be the most appropriate acquisition mode.

2.3.3.2 Optimised DIA method

For the purposes of targeted screening, the CE spread, pre-cursor ion and DIA reference ions were optimised as highlighted in Table 2.12 for the lipids and Table 2.13 for the corticosteroids.

Table 2-12: Optimised DIA method including CE spread and DIA reference ions for lipids (asterisks indicate the quantitative ion)

Compound	**Pre-cursor ion (*m/z*)**	**Optimised CE spread (eV)**	**DIA reference ions (*m/z*)**
6-Keto Prostaglandin $F_{1\alpha}$	373.2517	21.0-25.0	249.2147*
			167.1367
			211.1268
			319.2196
			205.1587
Thromboxane B_2	369.2268	9.0-13.0	169.0858
			195.1016*
			289.1808
			325.2013
Prostaglandin $F_{2\alpha}$	353.2323	21.0-25.0	193.1224*
			309.2058
			247.2057
			291.1952
			165.1273
Prostaglandin E_2	351.2159	12.0-16.0	271.2056*

			315.1953
			333.2060
			189.1273
11-Dehydro Thromboxane B_2	367.2104	18.0-22.0	305.2111*
			243.2107
			161.1321
			275.1060
			321.1121
Prostaglandin D_2	351.1044	12.0-16.0	271.2054*
			315.1952
			189.1274
			233.1171
Leukotriene D_4	497.2680	10.0-14.0	301.2140
			189.1626*
			319.2258
			283.2057
Leukotriene E_4	440.2452	10.0-14.0	301.2146
			189.1627*
			319.2244
			283.2033
Leukotriene B_4	335.2211	18.0-22.0	195.1016*
			129.0547
			123.0805
			151.1114
			203.1800
9-HOTrE	293.2113	10.0-30.0	275.1999
			171.1027*

			121.1014
			185.1161
18-HEPE	317.2109	10.0-30.0	255.2107
			299.2005
			215.1794*
			259.1690
15(S)-HETE	327.2770	15.0-19.0	257.2262
			301.2159
			175.1480
			219.2182*
			203.1794
			113.0965
17-HDHA	343.2283	10.0-30.0	281.2271
			201.1630
			245.1550*
			147.1181
			109.1023
13-HDHA	343.2265	10.0-30.0	281.2264
			193.1220*
			121.1017
			221.1171
12(S)-HETE	319.2271	10.0-30.0	179.1067*
			301.2154
			257.2261
			208.1091
			163.1136
5(S)-HETE	319.2263	12.0-16.0	257.2263

			301.2161
			115.0391*
			203.1796
15-HEDE	323.2577	10.0-30.0	305.2474
			223.1698*
AEA	348.2881	19.0-23.0	287.2357*
			203.1785
			133.1001
			245.2253
			269.2248
			119.0846
OEA	326.3040	19.0-23.0	309.2274*
			121.1002
			135.1156
			95.0848
Arachidonic Acid	303.2320	15.0-19.0	259.2405*
			205.1947
			177.0906
6-Keto Prostaglandin $F_{1\alpha}$-D_4	373.2517	21.0-25.0	249.2147*
Thromboxane B_2-D_4	373.2523	11.0-15.0	245.1901*
Prostaglandin $F_{2\alpha}$-D_4	357.2573	11.0-15.0	197.1474*
Prostaglandin E_2-D_4	355.9422	11.0-15.0	275.2305*
Prostaglandin D_2-D_4	355.1539	15.0-19.0	275.2305*
Leukotriene D_4-D_5	502.2994	14.0-16.0	194.1948*
Leukotriene B_4-D_4	339.2428	18.0-22.0	197.1140*
14(15)-DiHET-D_{11}	348.3060	10.0-30.0	207.1378*
15(S)-HETE-D_8	327.2767	12.0-16.0	226.1818*

12(S)-HETE-D_8	327.2761	10.0-30.0	184.1392*
5(S)-HETE-D_8	327.2776	18.0-22.0	116.0463*
OEA-D_4	330.3283	19.0-23.0	313.3015*
Arachidonic Acid-D_8	311.2817	12.0-16.0	267.2915*

Table 2-13: Optimised DIA method including CE spread and DIA reference ions for corticosteroids (asterisks indicate the quantitative ion)

Compound	**Precursor Ion (*m/z*)**	**Optimised CE spread (eV)**	**DIA reference ions (*m/z*)**
Hydrocortisone	363.2166	19.0-23.0	121.0642*
			327.1953
			309.1849
			267.1742
Cortisone	361.2010	22.0-26.0	163.1111*
			343.1911
			265.1589
			121.0641
18-Oxocortisol	377.1959	22.0-26.0	267.1744*
			285.1854
			121.0643
18-Hydroxycortisol	379.2115	22.0-26.0	267.1743*
			285.1850
			121.0645
			249.1629
11-Deoxycortisol	347.2217	22.0-26.0	109.0646*
			329.2110
			311.2005
			97.0645
Hydrocortisone-D_4	367.2417	22.0-26.0	121.0644*

2.3.4 Developed Surrogate Matrix

Due to the endogenous nature of the corticosteroids and lipids monitored, a surrogate matrix was used to mitigate as much of the endogenous lipids and corticosteroids present in equine plasma as possible to reduce the baselines and improve low-level quantification. The comparison was made between blank equine plasma that has undergone routine laboratory testing and did not present any exogenous drug administration with equine blank plasma that underwent LLE using DCM:EtOH (90:10 v/v). Comparisons were made for all the lipids and corticosteroids presented in Table 2.14.

Table 2-14: Surrogate matrix comparison between equine blank plasma and equine plasma after LLE using DCM:EtOH (90:10 v/v)

Compound of Interest	Area response from blank equine plasma	Area remaining after DCM:EtOH (90:10 ratio)	% Removed
6-Keto Prostaglandin $F_{1\alpha}$	2676	2592	3
Thromboxane B_2	8825	1330	85
Prostaglandin $F_{2\alpha}$	6841	5524	19
Prostaglandin E_2	3376	0	100
11-dehydro thromboxane B_2	9293	574	94
Prostaglandin D_2	5804	565	90
Leukotriene D_4	692	0	100
Leukotriene E_4	589	0	100
Leukotriene B_4	24129	666	97
15(S)-HETE	149308	838	99
5(S)-HETE	780443	41758	95
AEA	3511	1246	65
OEA	113580	10067	91

Arachidonic Acid	504190	18175	96
Hydrocortisone (HC)	1025062	174075	83
Cortisone (C)	16613	2147	87
18-oxocortisol	1771	1460	18
18-hydroxycortisol	2977	1412	53
11-deoxycortisol	6980	938	87

14 compounds had a percentage removal of over 80% whilst 5 compounds had a percentage removal less than 80%. With LLE being effective for more than 50% of the compounds, the wash-up of the endogenous lipids and corticosteroids using DCM:EtOH (90:10 v/v) was used for the preparation of the surrogate matrix for method validation and calibration spikes.

2.3.5 Method Validation for Selected Lipids and Corticosteroids

Method validation for the 100 µL method was completed for OEA, AEA, hydrocortisone and cortisone using the DCM:EtOH (90:10 v/v) surrogate matrix due to the endogenous compound present as seen in Table 2.14. These four biomarkers were chosen as they were the biomarkers consistently present throughout the administrations analysed in this project and showed the most potential change from the pilot study using the first administration of Triamcinolone Acetonide. The lipid biomarkers of OEA and AEA were chosen as they are analogues of each other and the pre-cursor for the AA cascade, whilst HC and C were also chosen as previous literature indicated the change in them was most prominent for a triamcinolone acetonide administration[93]. As majority of the endogenous analytes have been removed, the assumption is made that concentrations are accurate without the need for further corrections. This assumption was made for all compounds being validated even with AEA only having 65% removed. This is due to the amount present remaining in the matrix being very low in addition to the method allowing for numerous endogenous compounds to be removed instead of only one compound being completely removed from the matrix. Concentrations were set at 1 ng/mL for AEA, 5 ng/mL for OEA and C and 50 ng/mL for HC for precision, accuracy, recovery, and matrix effects. The concentrations of 5 ng/mL for C and 50 ng/mL for HC were chosen as these were previously validated for a method utilising 2 mL of

equine plasma[93]. For the lipid biomarkers of AEA and OEA, from early and unpublished profiling during optimisation, the concentrations of 1 ng/mL and 5 ng/mL were indicative of the levels of these biomarkers in equine plasma.

2.3.5.1 Linearity

Table 2.15 indicates the R^2 value using data obtained from 24th of January 2023 to 14th of April 2023 (4 batches). Figures A1 to A4 in appendices shows the overlayed calibration curve for the 4 weeks for OEA, AEA, hydrocortisone and cortisone respectively.

Table 2-15: R^2 values for AEA, OEA, cortisone and hydrocortisone

Compound	**Equation**	**R^2 value (averaged)**	**R^2 value (from instrument on 24th of January 2023)**
AEA	y = 0.2274x – 0.048	1.00	0.999
OEA	y = 0.795x + 4.226	0.999	0.997
Cortisone	y = 0.0284x + 0.0793	0.985	0.999
Hydrocortisone	y = 0.0186x + 0.2631	1.00	0.999

Further assessment of linearity was completed using the y-residuals to assess if there was any underlying bias in the calibration. Figure 2.4 demonstrate the residuals for AEA, OEA, cortisone and hydrocortisone and respectively. These provide no evidence of bias amongst the spiked samples due to the variability in the plots.

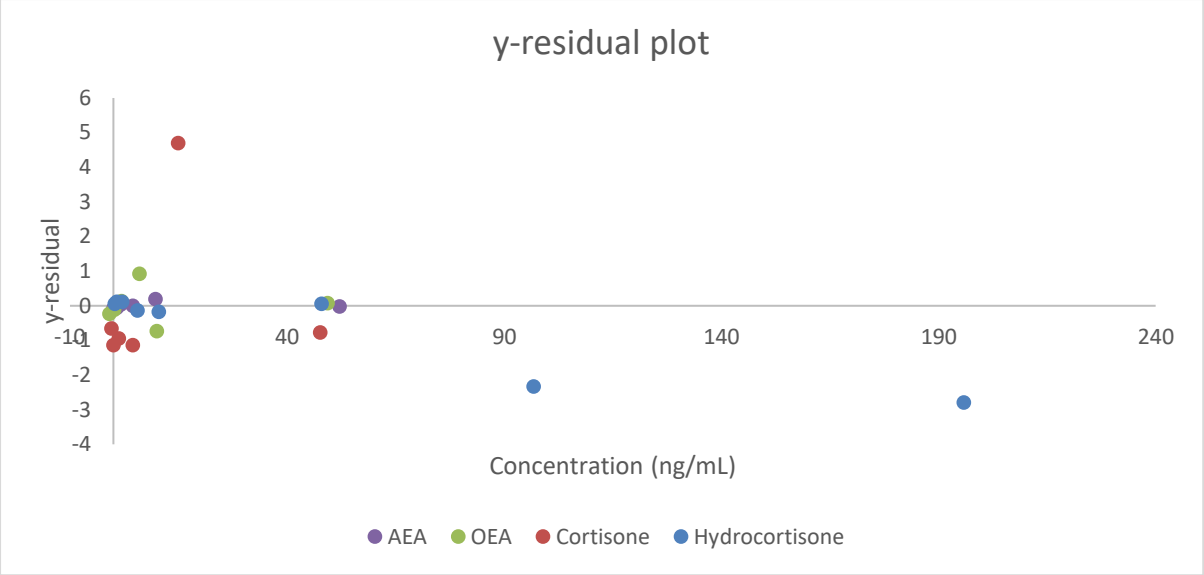

Figure 2.4: y-residual for AEA, OEA, cortisone and hydrocortisone in equine plasma over 4 separate batches

2.3.5.2 Sensitivity

From the concentrations of 0.4, 0.3, 0.2 and 0.1 ng/mL spiked in surrogate matrix as described in chapter 2.2.7.2, the LOD for AEA and cortisone was estimated to be 0.1 ng/mL while the LOD for hydrocortisone and OEA was estimated to be less than 0.1 ng/mL. The estimation is due to the inability to spike more accurately below 0.1 ng/mL in 100 µL of surrogate matrix in addition to the presence of some endogenous material still present in the surrogate matrix plasma. The LOQ for all 4 compounds was estimated to be 0.2 ng/mL.

Compared to previous literature, the LOD was < 0.05 ng/mL (for HC) and 0.05 ng/mL (for C) whilst LOQ was < 0.05 ng/mL (for HC) and 0.10 ng/mL (for C)[93]. With the acquired LOD and LOQ from the 100 µL method, it is evident with the decreased amount of sample volume, the sensitivity has decreased, which is consistent with the instrumentation and method utilised.

2.3.5.3 Accuracy, Precision, Recovery and Matrix Effects

Table 2.16 indicates the results for accuracy and precision relative to the criteria equal to or less than 15%. The compounds of OEA, HC and C were within the acceptable precision whilst AEA was not at a calculated RSD of 18%. Regarding accuracy, both AEA and HC showed high

relative error. Reasons for this could include the influence of endogenous substance in the surrogate matrix, extraction bias and inconsistent spiking between the replicates for a lower accuracy whilst all compounds show consistent ion suppression due to the ME being lower than 100%. Due to the inability to accurately quantify HC and accurately or precisely quantify AEA, these two compounds will only be interpreted through estimations or qualitative processes.

Comparatively between the 100 µL method and the 2 mL method previously established for HC and C[93], precision and matrix effects were consistent however, accuracy and recovery were not. As seen in table 2-16, precision and matrix effects for HC and C were similar however, accuracy and recovery were not consistent between the two methods. The difference in accuracy and recovery could be due to the decreased sensitivity of the instrument on the LC-HRMS compared to the triple quadrupole instrument in addition to the use of 20 x less sample volume as used for the published literature.

Table 2-16: Accuracy, precision, recovery, and matrix effects for AEA, OEA, cortisone and hydrocortisone comparing 100 µL and 2 mL method (n=7).

	AEA		**OEA**		**Cortisone**		**Hydrocortisone**	
Concentration (ng/mL)	1.00		5.00		5.00		50.0	
Method volume (µL)	100	2000	100	2000	100	2000	100	2000
Accuracy (% RE)	68.4 ± 0.76	25.0 ± 0.77	2.00 ± 0.86	7.70 ± 0.78	13.0 ± 0.77	5.20 ± 0.83	33.0 ± 1.29	11.0 ± 2.72
Precision (% RSD)	18.0 ± 0.79	5.70 ± 0.77	6.80 ± 0.91	4.90 ± 0.78	11.0 ± 0.82	8.00 ± 0.85	6.00 ± 1.71	6.70 ± 2.72
Recovery (%)	79.0 ± 0.77	46.0 ± 0.77	82.0 ± 0.77	25.0 ± 0.78	91.0 ± 0.76	50.0 ± 0.77	76.0 ± 1.95	58.0 ± 0.96
Matrix Effects (%)	93.0 ± 0.77	79.0 ± 0.76	71.0 ± 0.83	96.0 ± 0.76	62.0 ± 0.77	67.0 ± 0.76	72.0 ± 2.11	70.0 ± 0.78

2.3.5.4 *Stability*

Due to samples being stored in cooler bags and freezers prior to analysis, it was important to assess the stability of the chosen compounds in cooler environments. The stability study was completed over a 4-week period in duplicate with samples being extracted and analysed at the 2-week, 3-week and 4-week mark. Figures 2.5 to 2.8 displays the results of the stability study for the 4 compounds validated for temperatures of 4 °C or – 20 °C. All 4 compounds were deemed stable over the 4-week period in comparison to the time 0 concentration. For HC and C, this is consistent with previous literature as this time period of stability was shorter than the published stability of 3 months for equine plasma[93].

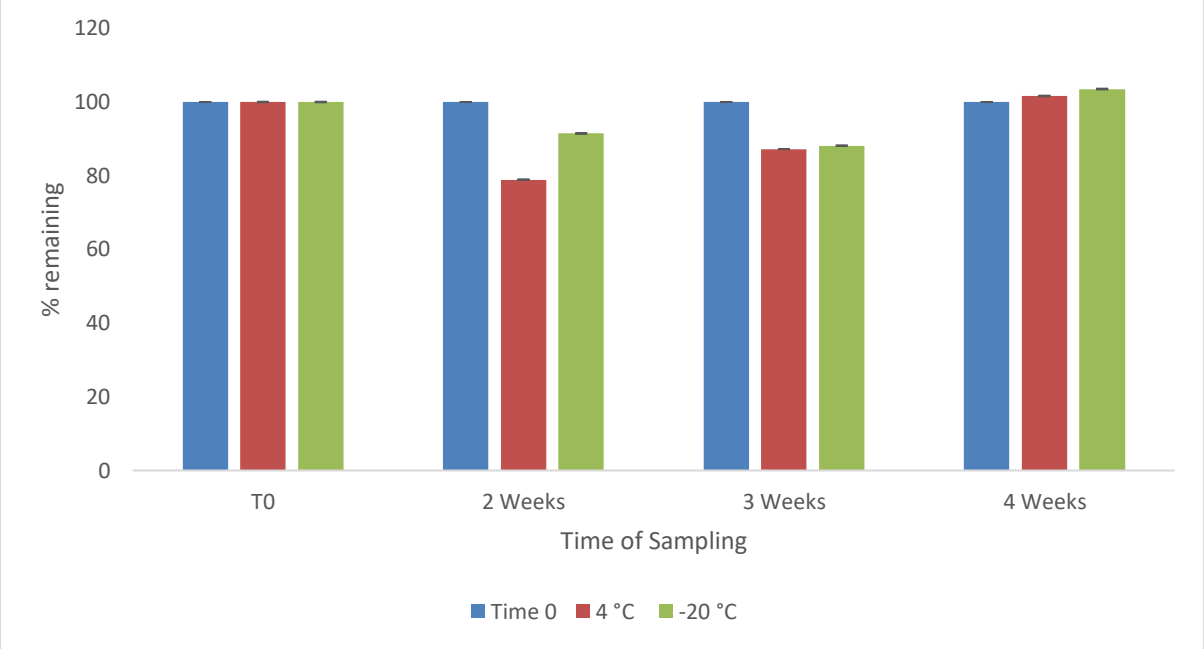

Figure 2.5: Stability results for AEA at 4 °C and – 20 °C (n=2).

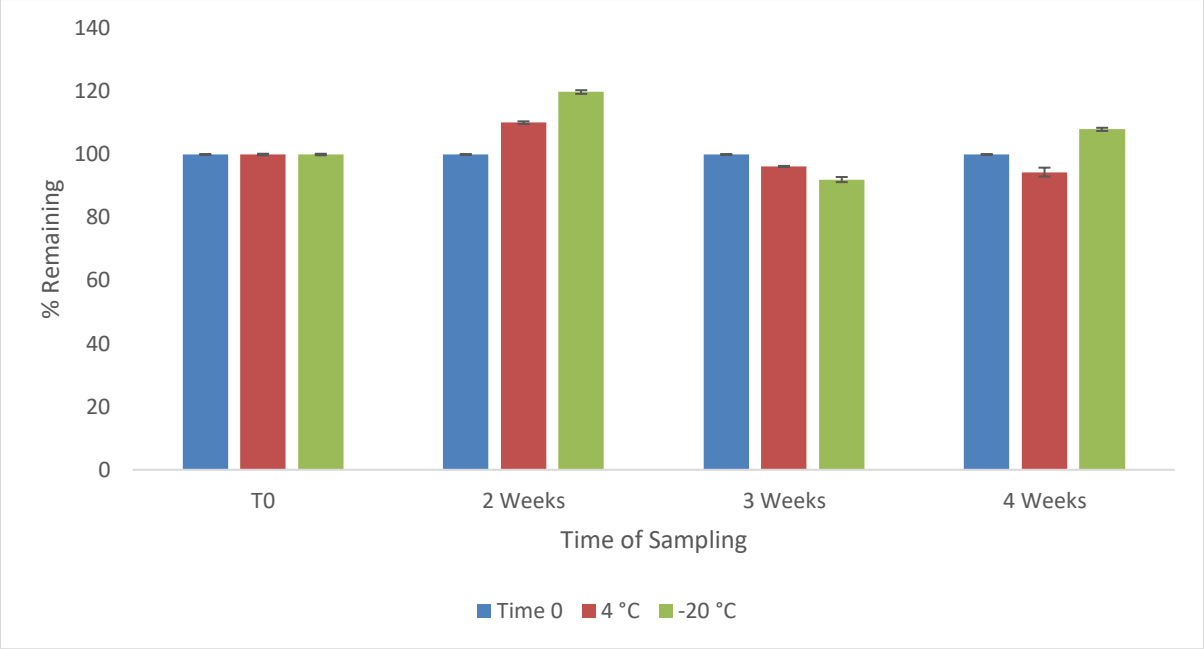

Figure 2.6: Stability results for OEA at 4 °C and – 20 °C (n=2).

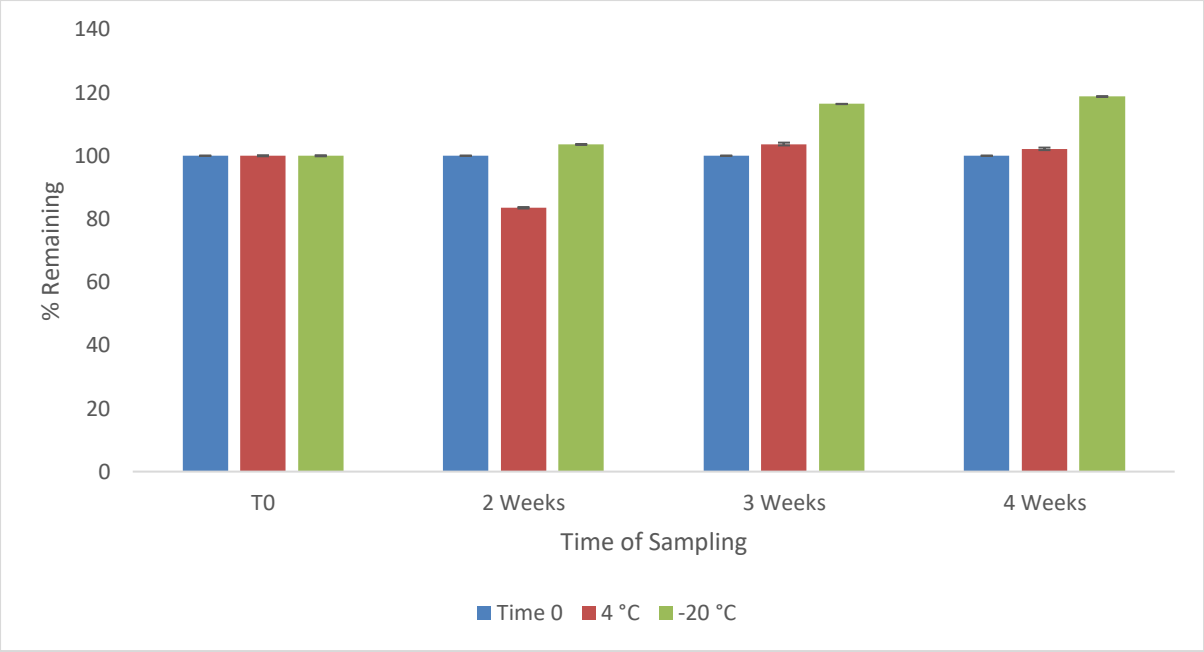

Figure 2.7: Stability results for cortisone at 4 °C and – 20 °C (n=2).

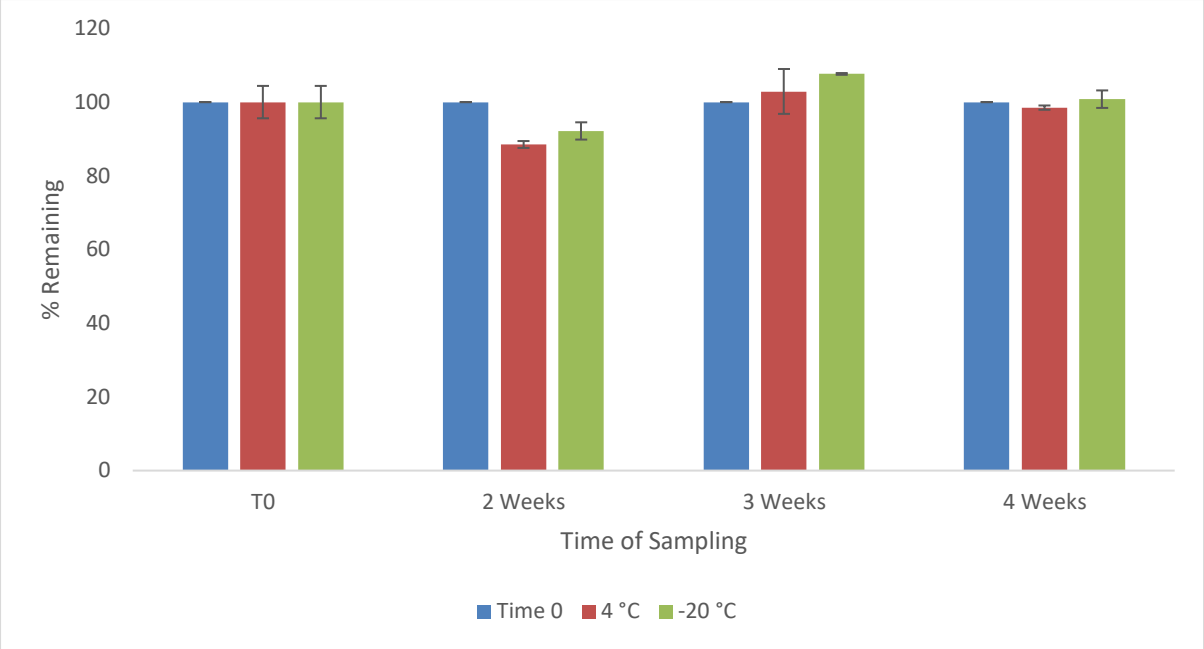

Figure 2.8: Stability results for hydrocortisone at 4 °C and – 20 °C (n=2).

2.4 Endogenous Biomarker Reference Population Results

A total of 750 samples were collected, extracted, and analysed for reference population statistics. From these 750 samples, all samples underwent one freeze thaw cycle. Following the reference population extractions, 33% of the lipid mediators had more than 50% of the samples above LLOQ and were quantifiable. Table 2.17 provides the percentage of the quantifiable concentrations of the lipid biomarkers.

Table 2-17: Percentage of detectable concentrations from the reference population study (n=750)

Compound	Number of samples > LLOQ	Number of samples < LLOQ	Percentage of detectable samples (%)
Hydrocortisone	750	0	100
Cortisone	750	0	100
Arachidonic Acid	654	96	87
11-deoxycortisol	632	118	84

17-HDHA	591	159	79
13-HDHA	566	184	76
5(S)-HETE	571	179	76
12(S)-HETE	492	258	67
AEA	365	385	49
OEA	317	433	42
15(S)-HETE	294	436	39
18-hydroxycortisol	219	531	29
Prostaglandin $F_{2\alpha}$	195	555	26
Prostaglandin D_2	127	623	17
18-HEPE	115	635	15
Prostaglandin E_2	50	700	6.7
9-HOTrE	33	717	4.4
11-dehydro thromboxane B_2	11	739	1.5
Leukotriene E_4	5	745	0.67
6-Keto Prostaglandin $F_{1\alpha}$	4	746	0.53
Leukotriene D_4	0	750	0
Leukotriene B_4	0	750	0
15-HEDE	0	750	0
18-oxocortisol	0	750	0

It should be noted that even if a compound was not detectable, it doesn't necessarily mean it is useless for this method. Different drugs may affect these compounds with the presence and absence being an important indicator of potential administration. The method was validated for AEA, OEA, cortisone and hydrocortisone for LOQs of 0.2 ng/mL for all biomarkers. As seen in Table 2.17, AEA was detected in 49% of samples whilst OEA was detected in 42% of samples indicating that these analytes are below quantifiable levels in approximately half the equine population using this method. As this study is the first study to

monitor AEA and OEA in the equine, it is unknown why these two compounds couldn't be effectively quantified however, it is hypothesised that this is likely due to the small volume (100 µL) of sample used. Hydrocortisone and cortisone were quantifiable in all samples analysed therefore, these four compounds will be further analysed and presented in this chapter. Further work will involve the validation of the remaining compounds.

Box plots were generated in MATLAB to assess if there were any significant differences between the three genders. Figures 2.9 to 2.12 highlights the boxplots for OEA, C, HC and HC/C values respectively.

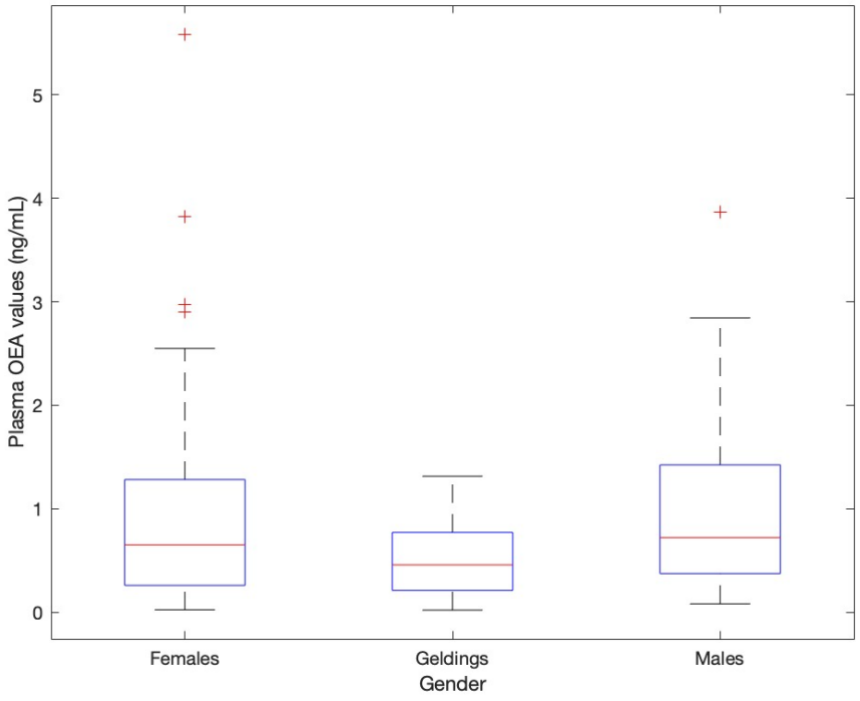

Figure 2.9: Box plot for the OEA gender comparison

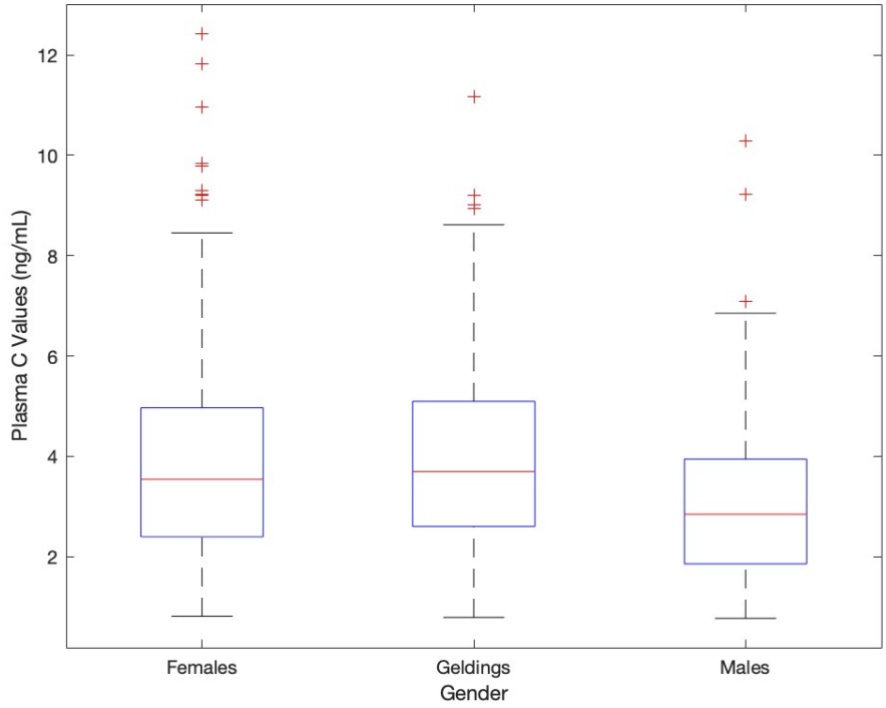

Figure 2.10: Box plot for the C gender comparison

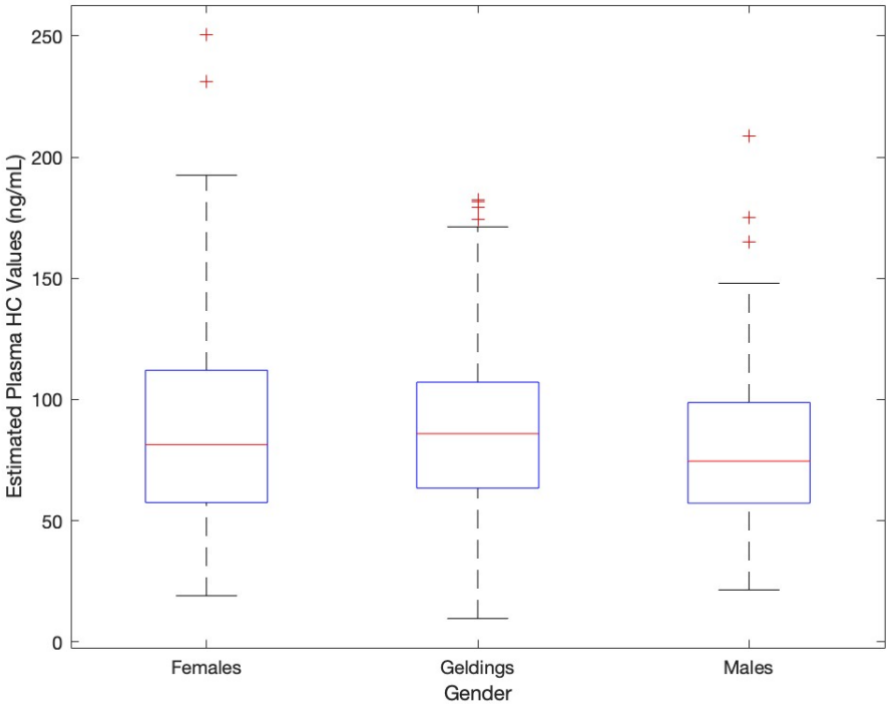

Figure 2.11: Box plot for the HC gender comparison

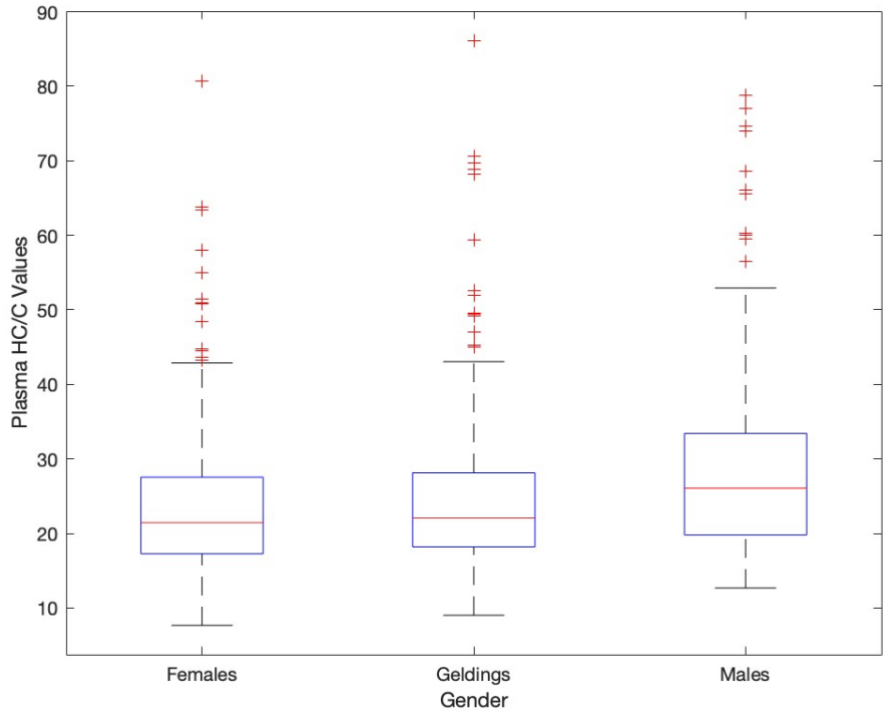

Figure 2.12: Box plot for the HC/C gender comparison

From the boxplots as seen in Figures 2.9 to 2.12, there does not appear to be a significant difference between the three genders for any of the biomarkers. It must be noted however, that there are limitations to this dataset with the major being that there is no repeated sampling from each horse and there is an unequal number of horses for each subgroup (the three genders). This is due to the lower number of stallions (males) racing in comparison to geldings and females resulting in lower sample submissions resulting always in an unequal number. A biostatistician could be consulted in the future to verify. Not withstanding this, from the boxplot conclusion, this allows for the genders for each of the biomarker sets to be combined to be used for any proposed thresholds or limits.

2.4.1 AEA

AEA concentrations remained consistent throughout the entire reference population however as the method could not be accurately or precisely quantitated, AEA concentrations are deemed as estimations rather than fully quantitative. From the 179 samples analysed for AEA (49% of all samples analysed for the reference population), estimated concentrations

ranged from a minimum concentration of 0.2 ng/mL to the maximum of 0.6 ng/mL (Figure 2.13) and a mean concentration of 0.39 ng/mL.

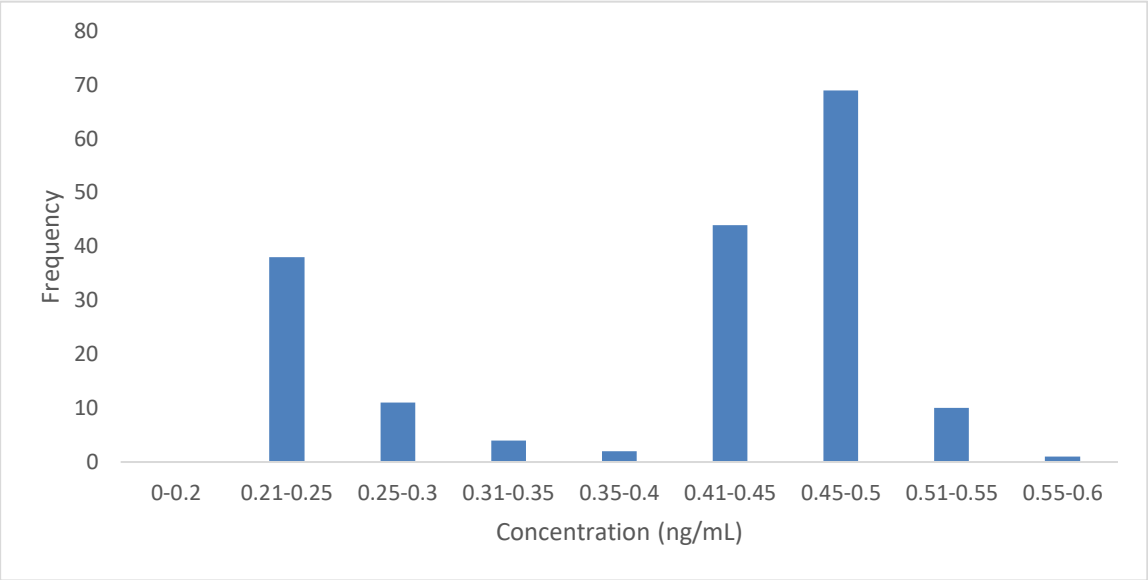

Figure 2.13: Frequency distribution of the estimated concentrations of AEA in equine plasma for all genders (n=179).

From the analysis of AEA concentrations in equine plasma, as statistical analysis was not completed, the estimated upper and lower thresholds of 0.2 ng/mL and 0.6 ng/mL are proposed from the range as seen in figure 2.13. Given that stability was not assessed with the population data, it is hard to determine whether extended periods of time would cause suppression of AEA in equine plasma. This doesn't necessarily indicate an administration of a doping substance (with AEA measuring effect), but a major limitation with this method is the limited sensitivity for low-level compounds. Further work exploring a larger volume is necessary to determine a more appropriate threshold. AEA has not been studied in the equine, therefore, comparisons with literature could not be completed. Cooperation with other interstate and international laboratories could determine if the concentrations obtained in this study was consistent with horses from other environments.

2.4.2 OEA

The measurable concentrations for OEA as seen in Figure 2.14 range from the LOQ of 0.2 ng/mL to 5.58 ng/mL. As OEA is an analogue of AEA, which is directly metabolised from AA, it

is likely the consistent presence is due to the it's high position on the AA cascade. The lower concentrations of OEA would be potentially due to the positioning of OEA on the AA cascade as the AEA analogue. As OEA is an endogenous cannabinoid receptor agonist, the stress factor could also be potentially affecting the properties OEA is responsible for[26,27].

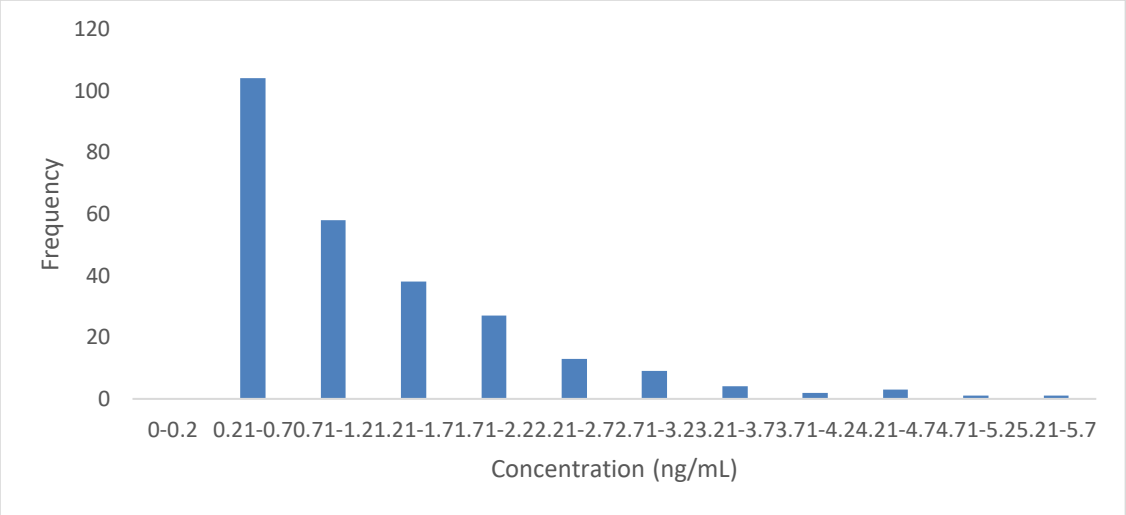

Figure 2.14: Frequency distribution for the concentrations of OEA in equine plasma for all genders (n=260)

From the reference population dataset, basal levels of lipids were determined with the possibility of proposing a suitable threshold. With the possibility of biomarkers being either parametric or non-parametric, a log normal transformation was completed on the dataset to determine if this followed a normal distribution[104]. Log transformation was completed on the data set with figure 2.15 showing the frequency distribution for these figures.

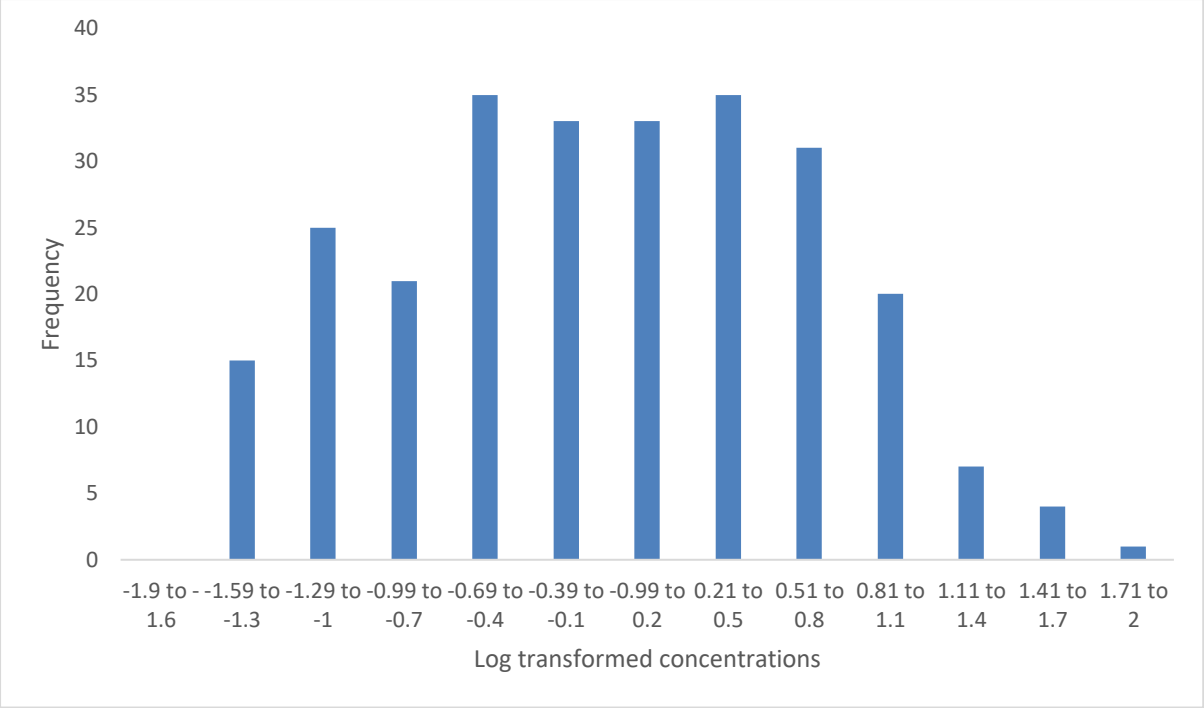

Figure 2.15: Frequency distribution for the log transformed concentration of OEA in equine plasma for all genders (n=260)

From figure 2.15, it can be seen that the log transformed values don't follow a normal distribution, therefore a normal probability plot was produced in *MATLAB* to determine if there was a parametric range from the log transformed values. The removal of outliers from the data set comprised of removing all results below LOQ of 0.2 ng/mL as data less than the LOQ can't be interpreted as accurate values. This was completed in accordance with the AORC guidelines[104]. Once the outliers were removed from the data set, Figure 2.16 shows the normal probability plot. According to this figure, the normal distribution range for OEA spans from log -1.17 to 0.49 equating to 0.31 ng/mL to 1.6 ng/mL. This resulted in 176 samples being deemed parametric which is 68% of the sample set.

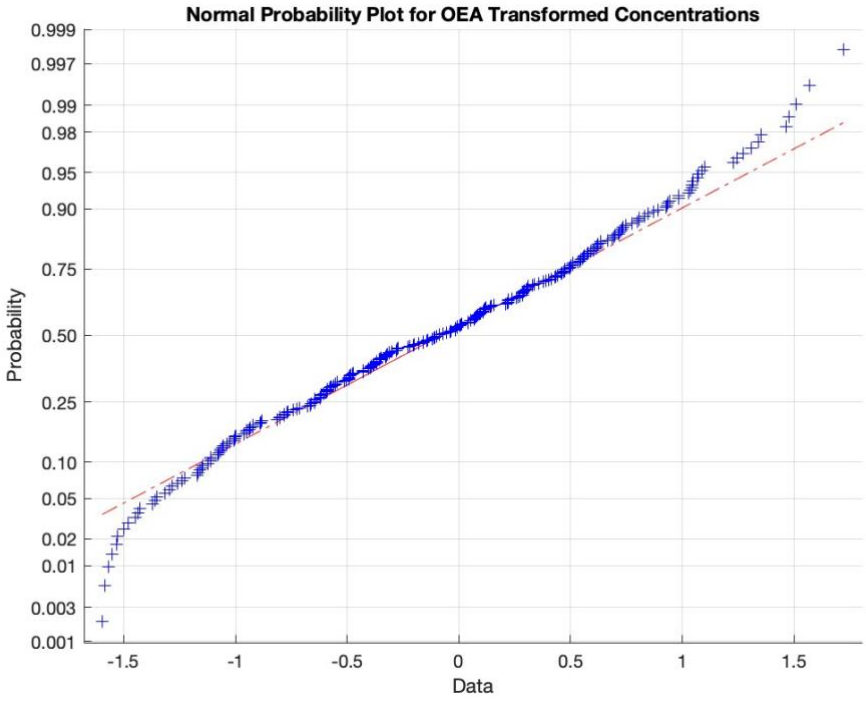

Figure 2.16: Normal probability plot for OEA log transformed concentrations (n=260)

Using the normal distribution range from the log transformed values, this dataset suggests a suitable threshold could be proposed for OEA concentrations using equation 2.7 in chapter 2.2.9. Using the mean, standard deviation and formulas, table 2.18 highlights the results from these steps.

Table 2-18: Results from the threshold equation for OEA in equine plasma using the parametric range.

Transformations	**Result**
Average log transformed concentration	-0.31
Standard deviation of log transformed concentration	0.48
Upper Threshold = Mean + (Standard Deviation x 3.72)	1.46
Lower Threshold = Mean - (Standard Deviation x 3.72)	2.09
Upper threshold (ng/mL)	8.1
Lower threshold (ng/mL)	4.3

The analysis of OEA concentrations in equine plasma using 176 samples, the equation 2.7 determined the thresholds of 8.1 ng/mL as the proposed upper threshold and 4.3 ng/mL as the proposed lower threshold for OEA. These values have been suggested with the assumption that there is no presence of therapeutics affecting OEA from the pre-race day samples that were being analysed. It must be noted that majority of the concentrations obtained from this method is below the lower threshold. This is most likely due to the 100 µL method not having the sensitivity necessary for OEA. The parametric calculations for the entire OEA data set are not appropriate given 433 samples were undetectable (below the LOQ). In addition to figure 2.15 not showing a normal distribution from the log transformed values for the detectable concentrations, a non-parametric workflow to determine the population reference limits was investigated, with table 2.19 highlighting the upper and lower threshold utilising non-parametric statistics. As this is in reference to non-parametric statistics, the original data set of 260 samples will be utilised.

Table 2-19: Results from threshold calculations for OEA in equine plasma using non-parametric statistics (n=260).

Calculations	**Population Reference Limit (ng/mL)**
Upper threshold	5.6
Lower threshold	0.21

From the non-parametric statistics, the upper and lower thresholds were calculated to be 5.6 ng/mL and 0.21 ng/mL. These concentrations appear to be more appropriate given the concentration range of OEA. Further work exploring a larger sample volume will need to be completed to compare whether this will increase the sensitivity of the method. Unfortunately, as OEA has not been studied in the equine, it is unknown whether these concentrations are consistent with other horses. It would be appropriate for interstate and international laboratories to conduct their own studies to determine if concentrations are similar.

2.4.3 Hydrocortisone

Hydrocortisone concentrations as expected from previous literature[93], was estimated in all samples ranging from 9.6 ng/mL up to 251 ng/mL in equine plasma. There are various possibilities for this large range with the most likely scenario being the differences in individual levels of hydrocortisone for each horse. Another scenario would be different horses being in varying circadian rhythms which is known to affect the levels of hydrocortisone in horses[80]. Different horses are subject to a varying amount of stress prior to racing which can also affect the hydrocortisone levels of each horse.

Given there is prior knowledge of the levels of hydrocortisone in 2 mL of equine plasma[93], the aim of the 100 µL method is to estimate if similar results could be seen from 20 times less sample. Ideally, the sample amount will follow a similar parametric distribution to the 2 mL sampling allowing for the proposal of upper and lower thresholds using parametric distribution. Figure 2.17 highlights the normal probability plot created in *MATLAB* displaying the parametric range for hydrocortisone.

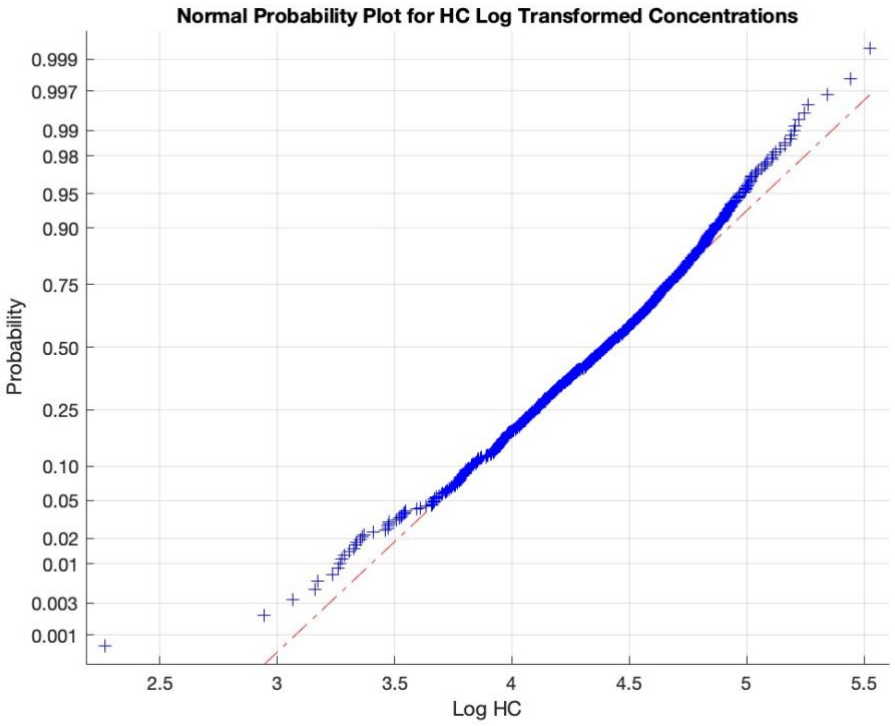

Figure 2.17: Normal probability plot for HC log transformed concentrations (n=750)

In Figure 2.17, there appears to be a parametric range from log 3.65 to log 4.72 equating to between 38 ng/mL and 112 ng/mL. This became a total count of 558 samples indicating that 74% of the samples analysed follow a parametric distribution. Using this parametric range from the log transformed values, the dataset can be used to estimate a threshold for HC equations using 100 µL of equine plasma. Using the equation 2.7 in chapter 2.2.9, the mean, standard deviation and formulas, Table 2.20 indicates the results from these transformations.

Table 2-20: Results from the threshold equations for HC in equine plasma (100 µL) using the parametric range.

Transformations	**Result**
Average log transformed concentration	4.3
Standard deviation of log transformed concentration	0.3
Upper Threshold = Mean + (Standard Deviation x 3.72)	5.3
Lower Threshold = Mean - (Standard Deviation x 3.72)	-3.3
Upper threshold (ng/mL)	200
Lower threshold (ng/mL)	0.2

From the 750 samples analysed for HC concentrations using 100 µL of equine plasma, the equation 2.7 estimates the upper threshold to be 200 ng/mL and the lower threshold at 0.2 ng/mL (LOQ) as the calculated lower threshold is below the LOQ. These figures have been estimated with the assumption of no known therapeutics affecting the levels of hydrocortisone.

2.4.4 Cortisone

The concentrations of cortisone (C) similar to previous literature[93] remained fairly consistent throughout the entire reference population not exceeding 13 ng/mL for all samples. Following a similar process to the other biomarkers previously explored, cortisone was analysed to determine whether or not it followed a parametric distribution after a log transformation. Figure 2.18 highlights the normal probability plot generated from *MATLAB* displaying the range of concentrations that fall within a parametric distribution.

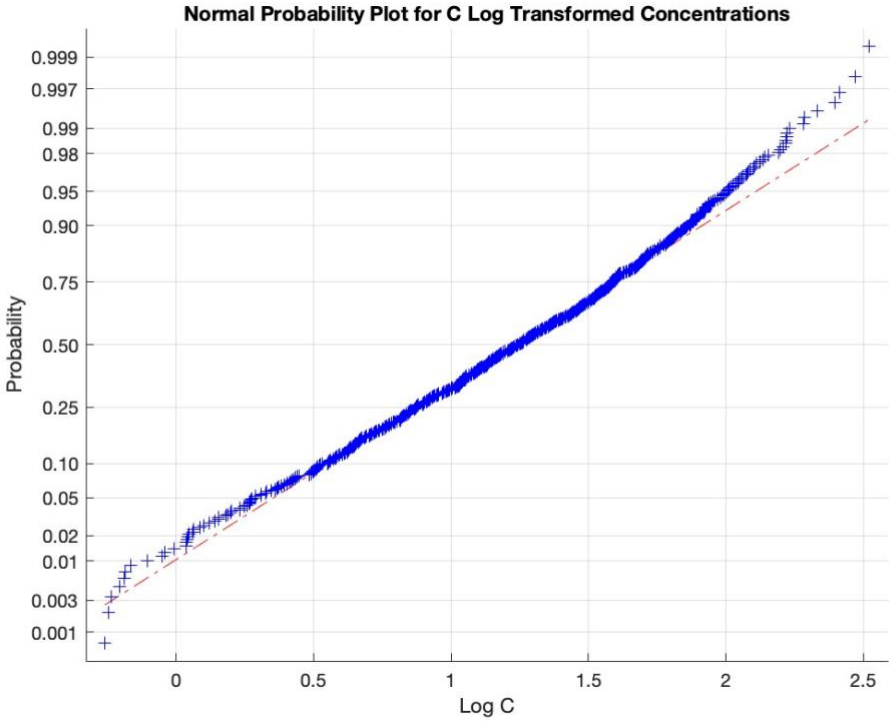

Figure 2.18: Normal probability plot for C log transformed concentrations (n=750)

Similar to the HC concentrations, C concentrations appear to follow the parametric distribution from log 0.48 to log 1.67 equating to between 1.6 ng/mL and 5.3 ng/mL respectively. This represented 537 samples which is equivalent to 72% of the data set being parametric. Using this parametric set from the log transformed values, the data can be used to propose a threshold utilising parametric equations 2.7 such as those found in chapter 2.2.9. Using the mean, standard deviation and relevant formulas, Table 2.21 highlights the results from these numerous transformations.

Table 2-21: Results from the threshold equation for C in equine plasma using the parametric range.

Transformations	**Result**
Average log transformed concentration	1.1
Standard deviation of log transformed concentration	0.3
Upper Threshold = Mean + (Standard Deviation x 3.72)	2.3
Lower Threshold = Mean - (Standard Deviation x 3.72)	0.04

Upper threshold (ng/mL)	10
Lower threshold (ng/mL)	1.0

From the analysis of C concentrations in 537 equine plasma samples, the proposed threshold equations for a parametric distribution equated the upper threshold to be 10 ng/mL and a lower threshold of 1 ng/mL. Given the stability of C, it would be extremely useful to note that any deviation from the limits would be highly suggestive of an exogenous drug being administered.

2.4.5 Hydrocortisone to cortisone ratio

Following the consistent and stable results of cortisone and in conjunction with previous literature[93], the hydrocortisone to cortisone ratio (HC/C) can be another biomarker to monitor in equine plasma. This ratio has been previously investigated using 2 mL of equine plasma[93]. Following a similar process to all the other biomarkers explored, the ratio values underwent a similar process to estimate a possible upper and lower threshold for 100 µL of equine plasma. Figure 2.19 displays the normal probability plot to determine the parametric distribution range following a logarithmic transformation.

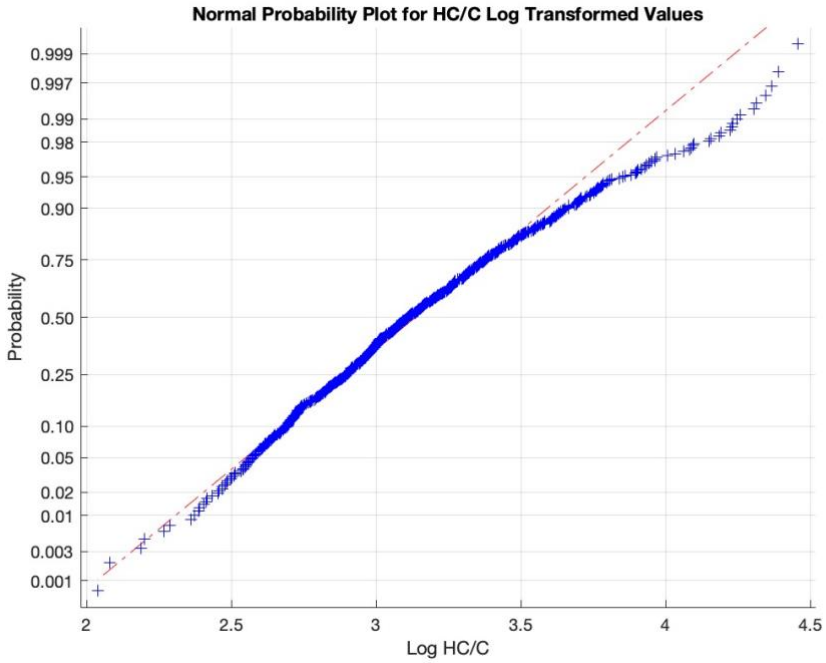

Figure 2.19: Normal probability plot for HC/C log transformed concentrations (n=750)

Similar to hydrocortisone and cortisone, the parametric distribution range for the log transformed values is from log 2.20 to log 3.40 which equates to between 9.0 to 30 respectively. From this range, a total of 576 samples were within the parametric range equating to 77% of the values. This percentage is higher than that of individual hydrocortisone and cortisone concentrations highlighting the advantage of using the ratio in comparison. Using the parametric range from the log transformed values, the dataset can estimate a threshold for HC/C using 100 µL of sample. Table 2.22 indicates the results from these calculations.

Table 2-22: Results from the threshold equation for HC/C in equine plasma (100 µL) using the parametric range.

Transformations	**Results**
Average log transformed concentration	2.99
Standard deviation of log transformed concentration	0.248
Upper Threshold = Mean + (Standard Deviation x 3.72)	3.92
Lower Threshold = Mean - (Standard Deviation x 3.72)	-2.07
Upper ratio threshold	50.3
Lower ratio threshold	0.12

From the analysis of the HC/C values in equine plasma using 750 samples from the 100 µL method, the estimated upper threshold calculated to be 50 whilst the estimated lower threshold was calculated to be 0.12. These figures are relatively consistent to those published for the 2 mL method where the proposed upper threshold is 58 and the proposed lower threshold is 0.24[93]. This highlights the robustness of HC/C, and the methods utilised to extract HC/C attaining similar results. These limits, in addition to the other biomarkers analysed, would be useful in the racing industry as complementary markers to potentially indicate the administration of exogenous drugs or therapeutics.

2.5 Equine Biological Passport – Longitudinal Profiling

The equine biological passport as stated previously is considered an intelligence model to be able to longitudinally monitor horses and their individual level of biomarkers present in the body. As an individual horse's concentration of markers can be inconsistent to those of the general population thresholds, it is important to establish individual reference limits (IRLs). These IRLs aim to detect anomalies in biomarker levels especially in horses that may exceed population-based thresholds due to natural variation. Using equations 2.8, 2.9 and 2.10 in chapter 2.2.10, three horses were selected for profiling using HC/C. Horse 1 is male, horse 2 is a female and horse 3 is gelding but since it has been demonstrated in this work that there is no gender difference using HC/C, a combined HC/C data set (combining the genders from the 100 µL data set) has been used for the reference population statistics. Figure 2.20 shows the profile for horse 1 and Figure 2.21 highlights the profile for horse 2 and Figure 2.22 highlights the profile for horse 3.

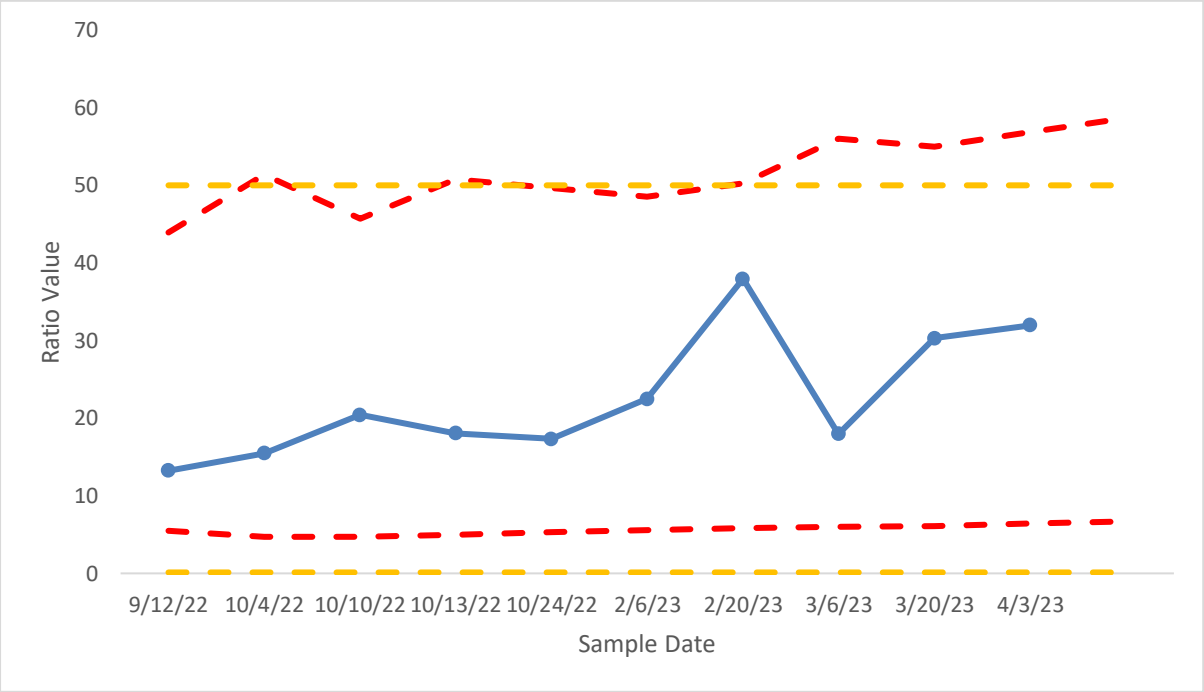

Figure 2.20: EBP profile for horse 1. Plasma HC/C values (blue line), upper and lower IRLs (red dotted lines) and upper and lower PRLs (yellow dotted lines).

As seen for horse 1 in figure 2.20 with a total of 10 samples from the 12th of September 2022 to the 3rd of April 2023, there appears to be no abnormal results exceeding either the upper

or lower limit for the individual horse or in comparison to the general population. On the 20th of February 2023, a sample recorded a higher HC/C value in comparison to the other samples. Whilst this sample did not exceed either threshold, it comes to show the variation of HC/C levels within a horse and why the use of IRLs would be advantageous to monitor for anomalies in the natural variation of biomarkers. The following sample on the 6th of March returned to previous levels of HC/C but the upper IRL did increase close to the upper proposed PRL showing the adaptive nature of this model.

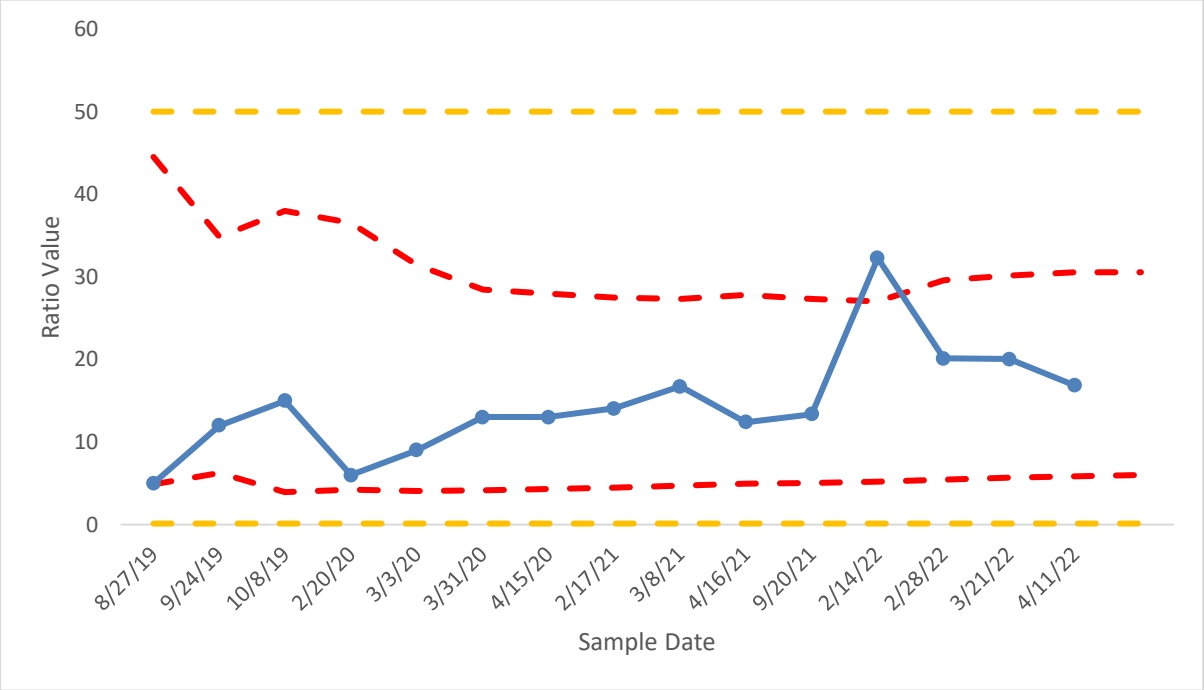

Figure 2.21: EBP profile for horse 2. Plasma HC/C values (blue line), upper and lower IRLs (red dotted lines) and upper and lower PRLs (yellow dotted lines).

Horse 2 in comparison as seen in Figure 2.21 showed greater variance in the HC/C values including an abnormal value at the 12th sample from the 14th of February 2023 (valued at 32) exceeding the upper IRL of 27 at that time. In comparison to the upper PRL of 58, this sample would not have been deemed abnormal without the use of the IRLs. This sample provided forensic intelligence to racing stewards for further investigations as to why this value was abnormal. This horse's values of HC/C return to levels below the IRL from the next sample on the 28th of February 2023 with the model adapting as such. This profile highlights the advantages of using IRLs in comparison to PRL, but this should not deter the use of PRLs as a more traditional form of doping control. Longitudinal profiling is not a form of confirmation

as it is only an intelligence model, therefore, more traditional approaches to confirmation are still necessary for any sanctions.

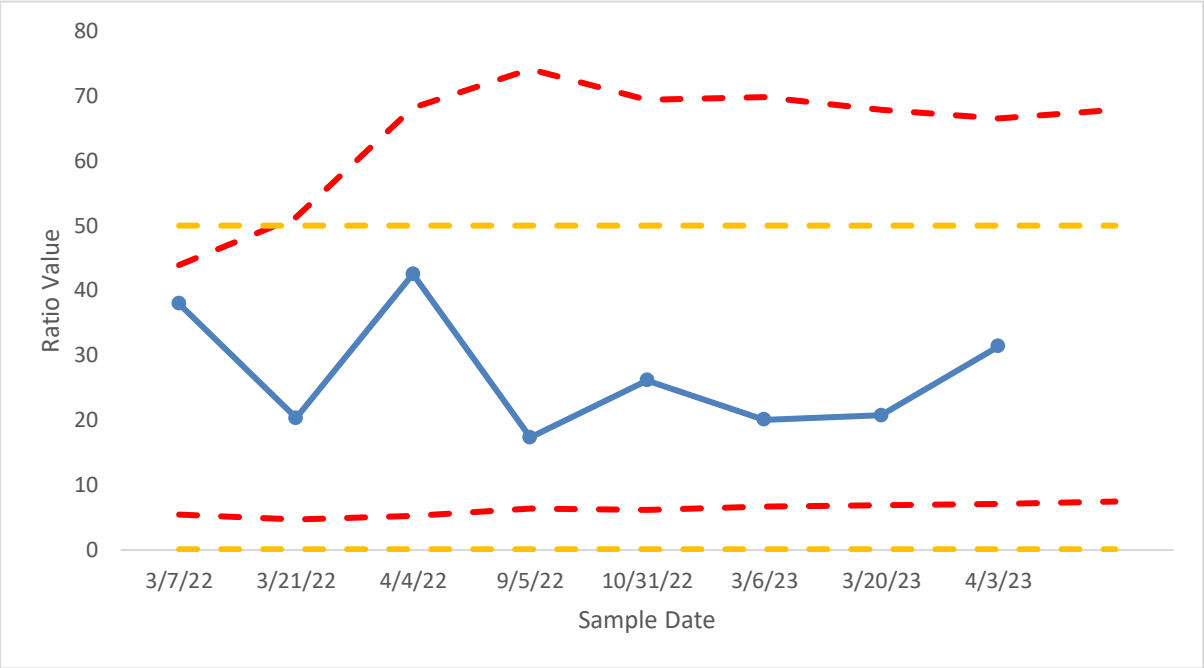

Figure 2.22: EBP profile for horse 3. Plasma HC/C values (blue line), upper and lower IRLs (red dotted lines) and upper and lower PRLs (yellow dotted lines).

Horse 3 (figure 2.22) showed greater variance in HC/C values with the IRLs exceeding PRLs. Whilst no sample was deemed abnormal, with the higher HC/C values, the individual horse itself is likely to have higher than normal HC/C values compared to the general population. In addition to the forensic intelligence provided from IRLs when sample are consistent, the intelligence is also important when the IRL exceeds the PRL. Whilst for horse 1 and 2, both IRLs do not exceed the PRL, in the case of IRLs exceeding PRLs, it is likely this is due to higher standard deviations between samples. This equates to a standard deviation of greater than 0.3 when it is seen that the IRL exceeds the PRL. This may indicate evidence of doping or another unknown health issue within the horse which can allow for further investigations from veterinarians and stewards. Nevertheless, the potential for the use of IRLs and profiles for biomarker assessment is an advantageous intelligence-based anti-doping strategy.

2.6 Conclusion

From the 25 lipids and endogenous corticosteroids extracted and analysed, 4 biomarkers underwent validation as representative biomarkers. The biomarkers of AEA and HC were to be deemed as estimations or to be interpreted qualitatively due to accuracy and precision criteria not being fulfilled. The biomarkers of OEA and C were validated successfully for full quantification. From the method validation, the biomarkers of AEA, OEA, C and HC had an estimated LOD of less than 0.1 ng/mL and a LOQ of 0.2 ng/mL.

Reference population data collated for this project totalled 750 samples extracted and analysed. Box and whisker plots were utilised to determine if there were significant differences between genders. From the visual comparison of the boxplots generated in *MATLAB* (Figures 2.9 to 2.12), these have indicated no significant differences between genders for all markers allowing for combined proposed population thresholds. From the samples analysed, C was deemed consistent with quantification not exceeding 13 ng/mL consistent with previously published literature[93]. From this finding, the proposed HC/C value was included as a biomarker for the 100 µL method. From the data sets, the following biomarkers were deemed parametric, C with 72%, HC/C with 77% of samples parametric and HC estimated to have 74% of samples parametric. This allowed for thresholds to be proposed using parametric transformations for population reference limits of 10 ng/mL and 1.0 ng/mL for C and 50 and 0.12 for the HC/C ratio. HC was estimated to have population reference limits of 200 ng/mL and 0.2 ng/mL. OEA had 56% of values parametric, whilst more than 50% of the samples, was evident that non-parametric statistics needed to be completed to ensure more of the sample values were used. From calculating the 0.005^{th} to 99.995^{th} percentile to determine the lower and upper limits the limits of 0.21 ng/mL and 5.6 ng/mL for OEA were proposed. Using the estimated range, the limits of 0.2 ng/mL and 0.6 ng/mL were proposed for AEA.

Utilising these thresholds, profiles for the EBP were generated using the HC/C values due to the ongoing analysis over the past few years. This resulted in three profiles being presented with horse 1 being a normal profile with no abnormal samples, horse 2 indicated an abnormal sample on the 14^{th} of February 2023 and horse 3 showed the IRLs being higher than PRLs

showcasing the use of profiles for horses with naturally higher variation of the biomarker. Suspicious profiles would undergo further investigation to determine the possible reasoning for the abnormal profile, but these profiles highlight the advantages of using the IRLs in comparison the PRLs. The assessment of the lipids and endogenous corticosteroids has allowed for a better understanding of how the biomarkers behave naturally.

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Chapter 3: Triamcinolone Acetonide Administration

3.1 Introduction

The need to monitor biomarkers has been evident for many exogenous drugs, in particular glucocorticoids due to their long-lasting and potent effects. A commonly used synthetic glucocorticoid in the equine industry is triamcinolone acetonide (TACA) that is approximately 5-times more potent than hydrocortisone (HC) as an anti-inflammatory agent and can be an effective analgesic for pain relief[108,109]. This can result in increased concentrations of anti-inflammatory mediators whilst decreasing the inflammatory mediators[62]. It is commonly used for the management of osteoarthritis in the horse which can be detrimental to the joints[108]. Currently in the rules of racing in Australia, AR 87 controls the use of intra-articularly administered corticosteroids by prohibiting their use for at least 8 days (192 hours) prior to racing[110].

The effects of TACA have been studied by *Mangal* et al with the authors exploring the inflammatory mediators in equine plasma. The authors utilised calcium ionophore (CI) and liposaccharide (LPS) stimulation as an *ex vivo* model for the eicosanoids in the presence of an exogenous drug[62]. Using the *ex vivo* models, CI stimulation was indicative of the LOX enzyme activity (15- and 5-LOX) whilst LPS stimulation was indicative of COX enzyme activity (COX-1 and COX-2) with Mangal's study looking at seven of the lipids in the arachidonic acid cascade (i.e. 5-HETE, LTB_4, 15-HETE, TxB_2, PGE_2, $PGF_{2\alpha}$ and 6-Keto $PGF_{1\alpha}$)[62]. From Mangal's study, TxB_2 PGE_2, $PGF_{2\alpha}$ and 6-Keto $PGF_{1\alpha}$, the major products expressed through COX-2 enzyme activity, were down-regulated, suggesting inhibition of the enzyme. Using CI stimulation, the concentration of 5-HETE was significantly increased however, for LTB_4, there was a significant decrease in concentration for a short period of time (6-8 hours post administration)[62].

This study concluded that major products expressed through the COX-2 enzymatic activity are down-regulated under the influence of an external glucocorticoid suggesting inhibition of the COX-2 enzyme [62]. There is speculation that PGE_2 has the potential to have a binary role in inflammation. This is potentially due to COX-1 producing PGE_2 in early phase inflammation for pro-inflammatory properties[62]. Comparatively, the delayed production of PGE_2 by the m-PGES-1 enzyme, coupled to COX-2 could additionally play a role in anti-inflammatory properties [62,68].

3.1.1 Aims

A lipidomic analysis on equine plasma was conducted under an administration of TACA. The aim of the TACA administration was to determine if the use of lipid and corticosteroid biomarkers could indirectly extend the detection time of the pharmacological effects of TACA in addition to the detection of the elimination of TACA itself. Any indirect detection time in addition to the current detection time for TACA could be advantageous in relation to the current AR 87 rule controlling the intra-articular use of corticosteroids.

3.2 Methods

3.2.1 Chemicals and Reagents

Please refer to chapter 2.2.1 .

3.2.2 Reference Materials

Please refer to chapter 2.2.2 for lipids.

For TACA, two sources of TACA were obtained for either the calibration or quality control. One was obtained from the Laboratories of the Government Chemist (Teddington, UK) and the second was obtained from NovaChem (Heidelberg West, VIC, Australia). The relevant internal standard for TACA was D_7-Triamcinolone Acetonide (D_7-TACA) which was purchased from Toronto Research Chemicals (North York, Ontario, Canada).

3.2.3 Sample Preparation/Lipid and Corticosteroid Extraction Method

Please refer to chapter 2.2.5 for the endogenous lipid and corticosteroid extraction method. TACA was also extracted using the same method.

3.2.4 Triamcinolone Acetonide (TACA) Administration Study

A 3-horse study (Horse 4, 5 and 6) was completed using a dose of 18 mg of Jurox Triamcinolone-Forte. This was injected intra-articularly (IA) into the fermoropatellar joint of

the horse. Blood samples were collected from 24 hours prior to the administration, 0 (pre-administration), 2, 5, 10, 20, 30, 40, 50, 60, 90 min, 2, 2.5, 3, 4, 6, 8, 12 hours, 1, 1.5, 2, 2.5, 3, 4, 5, 6-, 7-, 8- and 9-days post-administration. Blood samples were collected from the jugular vein using a catheter and stored in BD Li-Heparin 10 mL Vacutainers. Blood samples were immediately centrifuged at 1,500 x *g* for 10 minutes to obtain plasma which was then stored at -20 °C until analysis. Samples in this study did undergo one freeze thaw cycle prior to extraction and analysis. Animal ethics approval (A19365) was obtained for this study from Charles Sturt University Animal Care and Ethics Committee and reviewed by the Racing NSW Animal Care and Ethics Committee.

3.2.5 Instrument Conditions

Please refer to chapter 2.2.6 Instrument parameters.

3.2.6 Method Validation Preparations

Please refer to chapter 2.2.7 for the method validation parameters assessed and methods utilised for linearity, sensitivity, accuracy, precision, recovery, matrix effects and stability as outlined by Peters *et al*[99]. Concentrations for the TACA method validation parameters can be found in chapter 3.4.

3.2.7 Data Analysis Parameters

All data acquired on the LC-HRMS was processed using *Shimadzu Insight Explore* software (Version: 3.8 SP1) with further data processing completed using *Excel* (version 16.71) and MetaboAnalyst (version 5.0). For further information on the raw data, refer to chapter 2.2.8.1. For methods data processing the semi-targeted and non-targeted screening, please refer to chapter 2.2.8.2 Semi-Targeted Screening and 2.2.8.3 Non-Targeted Screening.

In addition to following the non-targeted screening data processing protocol as detailed in 2.2.8.3, a workflow was developed using the *Shimadzu Insight Explore* program utilising the naïve feature finding algorithm. This algorithm provides a list of pre-cursor ions that have an intense abundance and generate a list that contain the retention time, mass, and peak area.

This list is then transformed into a new data processing method through *Insight Explore* and the samples are further re-processed using the new list of generated compounds. Using the re-processed data, the data is taken from *Insight Explore* into *MetaboAnalyst* for statistical analysis. Statistical tests including principal component analysis (PCA), volcano plots and heat maps are utilised to determine the up and down-regulated compounds. Compounds that were putatively identified from these tests were taken back into the Insight Explore program and compared with the initial generated compound list to determine the mass of the compound. As the Explore software has an additional function for predicting the molecular formula, this was also noted provided the mass error was less than 5 ppm. Masses were grouped together based on the 3rd decimal place to be deemed similar.

3.3 Results and Discussion

3.3.1 Method Validation for TACA

Method validation was assessed for TACA using linearity, selectivity and specificity, sensitivity, accuracy, precision, recovery, matrix effects and stability using the concentration of TACA derived by linear regression in relation to the internal standard D_7-TACA.

3.3.1.1 Linearity

Calibration was done at 0.1 ng/mL, 0.2 ng/mL, 0.5 ng/mL, 1 ng/mL, 2 ng/mL, and 5 ng/mL. A 2000 ng/mL solution was prepared from the stock solution of 976.12 µg/mL with subsequent working solutions of 10 ng/mL, 1 ng/mL, and 0.5 ng/mL to spike into 100 µL of equine plasma. Table A4 in the appendices highlights the amounts spiked into 100 µL of equine plasma to prepare the calibrators.

The averaged R^2 value across 3 batches of calibration curves was determined to be 0.998 indicating a linear calibration curve. Residual analysis (n=3) showed no bias in the data through the random placements of the residual data points for each concentration (Figure 3.1)

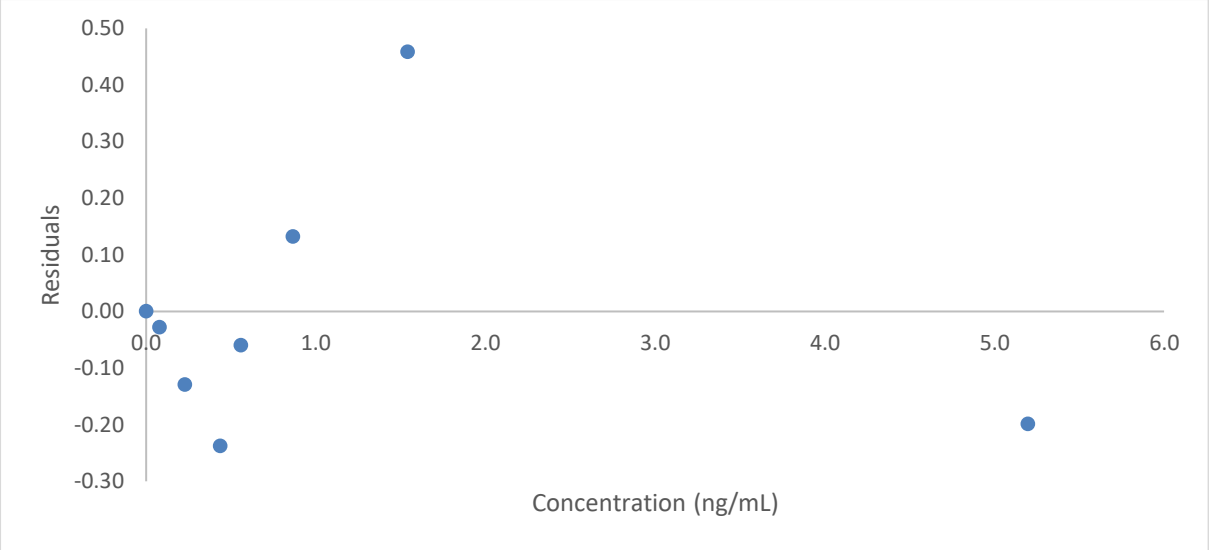

Figure 3.1: Average y-residual analysis of linearity assessment for TACA in equine plasma (n=3)

3.3.1.2 Selectivity and specificity

TACA was detectable at Rt 10.035 minutes with good peak shapes and baseline as seen in figure 3.2. TACA was monitored using the *m/z* of 479.2091 whilst the internal standard of D_7-TACA was monitored using the *m/z* of 486.2524.

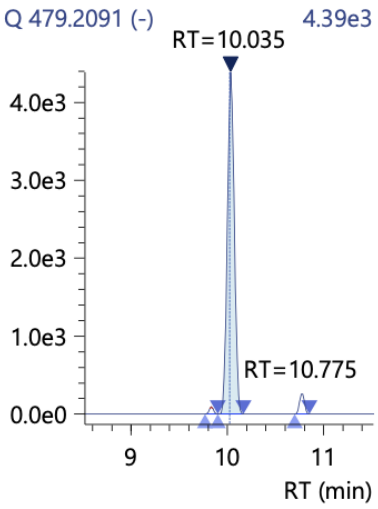

Figure 3.2: Chromatogram of TACA at 2 ng/mL in equine plasma

3.3.1.3 Sensitivity

TACA was analysed in both positive and negative ESI mode, therefore a separate limit of detection (LOD) and limit of quantification (LOQ) were established for each mode. LOD and LOQ was confirmed using replicates of 7. LOD and LOQ were estimated by a visual comparison for signal-to-noise (*S/N*) greater than 3 for LOD and 10 for LOQ. Table 3.1 summarises the LOD and LOQ for the respective acquisition modes.

Table 3-1: LOD and LOQ of TACA (n=7)

Acquisition Mode	LOD (ng/mL)	LOQ (ng/mL)
Negative	0.10	0.20
Positive	0.05	0.10

3.3.1.4 Accuracy, Precision, Recovery, Matrix Effects and Stability

Accuracy and precision were assessed as relative error (%RE) and relative standard deviation (%RSD) at the three-quality control (QC) levels in replicates of 7. QC levels chosen for TACA were 0.5 ng/mL as the low QC, 1 ng/mL as the medium QC and 2 ng/mL as the high QC. Precision and recovery were within the acceptable ranges for equine plasma being less than or equal to 15% for precision and above 60% for recovery. Matrix effects did indicate that at the LQC, there is evidence of ion enhancement however for MQC and HQC, there is evidence of an ion suppression being under 100%[102]. Accuracy was higher than the required relative error of 15% for all three QC levels, likely due to a bias from small co-eluting peaks from the sample matrix affecting the correct peak area integration. This will likely affect the accuracy of TACA determinations at such low concentrations from the low sample volume of 100 µL. If the concentrations were higher, the accuracy relative error might possibly reduce. Whilst these values for accuracy are high, it must be noted that one of the main objectives of this project is to determine whether biomarkers could complement the direct detection of an administration. Therefore, the detection of TACA is not the main objective. The method used is fit-for-purpose for longitudinal assessment of the biomarkers over time rather than a direct detection of the drug itself. Therefore, precision was deemed more important than accuracy. Regarding stability, TACA was deemed stable at -20°C for 6 months as analysed in previous

literature completed by Tou *et al* (chapter 2.1.1)[93]. Table 3.2 highlights the accuracy, precision, recovery, and matrix effects of TACA.

Table 3-2: Accuracy, precision, recovery, and matrix effects for TACA (n=7)

Quality Control Level	Accuracy (% RE)	Precision (% RSD)	Recovery (%)	Matrix Effects (%)
LQC (0.5 ng/mL)	52	15	93	103
MQC (1 ng/mL)	21	12	85	89
HQC (2 ng/mL)	34	9.8	73	88

3.3.2 Targeted Detection

3.3.2.1 TACA Elimination

An administration study of TACA was completed with quantification against D_7-TACA as an internal standard. A total of 3 horses had samples extracted and analysed for a period from one day prior to administration to 9 days post-administration. It must be noted that due to method validation criteria for accuracy not being achieved, the concentrations of TACA are estimations only. The elimination of TACA is shown in Figure 3.3 (for raw data, refer to table A5 in the appendices) highlighting a detection period between 2 minutes and 2.5 days post-administration. This time is consistent with previous studies conducted by Mangal *et al.* where the elimination of TACA was detected to be between 2 hours and 2 days post-administration [62]. Concentrations of TACA in the study conducted by Mangal were also slightly higher in comparison with the concentration at 2 h being 8.86 ng/mL in Mangal's study whereas in this study, concentration at 2 h did not exceed 2 ng/mL. This could be due to the higher amount of TACA being administered in the study conducted by Mangal (0.04 mg/kg: approximately 21.69 mg) in comparison to this study (18 mg). Alternatively, this could be due to the different route of administration resulting in different rates of drug absorption.

Other studies conducted on intra-articular administrations of TACA include those of Knych *et al*[109]*.,* Kay *et al*[111] and Chen *et al*[112]*.* The study conducted by Knych *et al* saw TACA levels fall below the LOQ of 0.1 ng/mL approximately 3 days post-administration which is a longer detection period compared to this study[109]. This could potentially be due to the dose (9 mg) and differing joint of administration with Knych using 9 mg in the right antebrachiocarpal joint whilst this study used 18 mg in the fermoropatellar joint. The study conducted by Kay *et al* saw TACA levels fall below the LOD 12 hours post-administration however using the same dose as Knych (9 mg)[111]. Kay's study also explored the use of multiple joints (not the fermoropatellar joint) and the ELISA for instrument analysis which potentially can account for the decreased sensitivity. The study conducted by Chen *et al* saw the use of the same dose of TACA with this study however injected into the intercarpal and tibiotarsal joints rather than the fermoropatellar joint[112]. Chen used serum instead of plasma concentrations with TACA not detectable 3 days post-administration using a radioimmunoassay method[112]. The results from Chen's study are consistent with this study that showed TACA levels not detectable 2.5 days post-administration. This suggests that the dose administered could indicate the time before TACA is undetectable independent of instrument and administering joint.

Other possibilities for the differences possibly include the administration route and the accuracy seen with the current study that only uses 100 µL of sample. Mangal utilised intra-venous administration and 1 mL of sample whilst this study was conducted via IA and using 100 µL of sample which may also account for the higher concentrations due to the systemic circulation of TACA in the system. This would also result in greater effects to the biomarkers especially during the earlier time points.

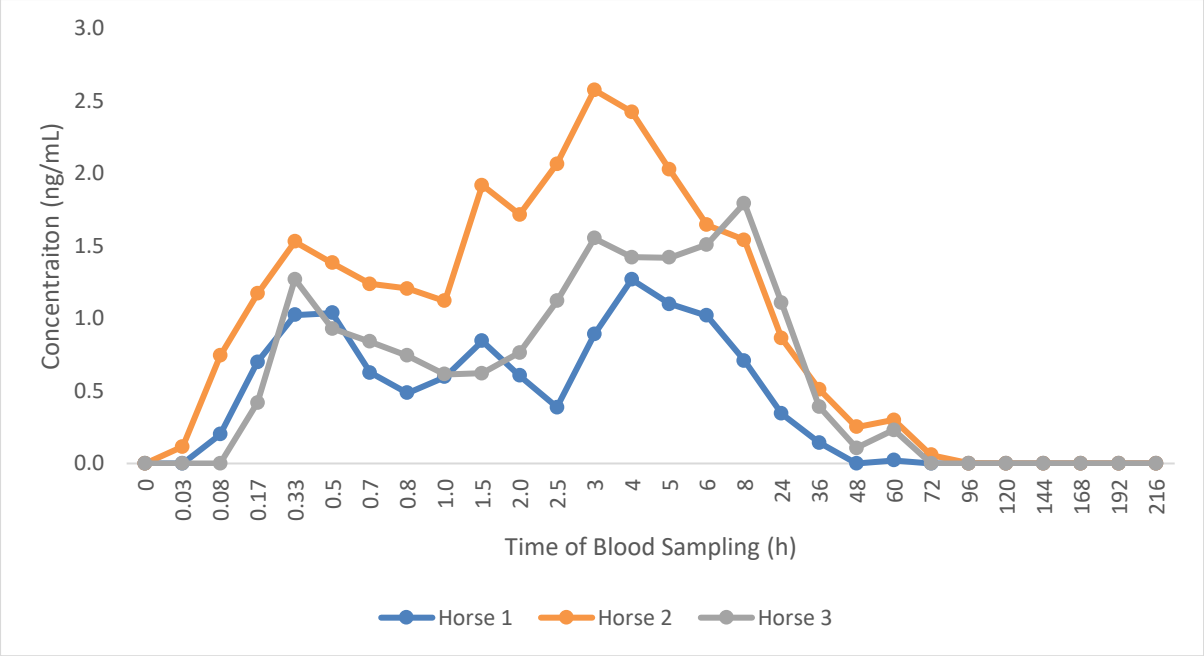

Figure 3.3: Elimination Curve of TACA for 3 horses in negative acquisition (Blue indicates horse 4, orange indicates horse 5 and grey indicates horse 6). All concentrations are deemed as estimates.

3.3.2.2 Lipid Detection

From 14 targeted lipids of 6-keto prostaglandin $F_{1\alpha}$, thromboxane B_2, prostaglandin D_2 (PGD_2), prostaglandin E_2 (PGE_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), leukotriene B_4, leukotriene D_4, leukotriene E_4, 11-dehydro thromboxane B_2, 15(S)-HETE, 5(S)-HETE, arachidonic acid (AA), arachidonoyl ethanolamide (AEA) and oleoyl ethanolamide (OEA), 8 lipids were detected in all 3 horses, 15(S)-HETE, 5(S)-HETE, AA, PGD_2, $PGF_{2\alpha}$, AEA and OEA. These lipids were chosen as a pilot study prior to including the rest of the lipids as noted in chapter 2.2.2. All biomarker concentrations (except for OEA and C) are deemed as estimations due to method validation not being performed or criteria not being met. In comparison with the study conducted by Mangal, $PGF_{2\alpha}$ and 15-HETE observed a significant reduction in concentration using LPS-treated equine whole blood (EWB) whilst 5-HETE observed a significant increase of concentration (using EWB) compared to baseline values[62]. It must be noted that Mangal's study utilised IV administration and LPS/CI treated EWB which may result in higher concentrations of TACA in the systemic circulation which will likely have a greater effect on the biomarkers (possibly earlier than intra-articular administration). Many of the lipids

detected in this administration primarily followed unchanged estimated concentration such as 15(S)-HETE, 5(S)-HETE, AA, PGD_2, $PGF_{2\alpha}$ and AEA. A summary table containing the concentrations obtained for the detected lipids at each time point administered can be found in tables A5 to A8 in the appendices.

The only lipid altered in the later time points of administration was OEA (raw data for OEA can be found in table A8 of the appendices) with an increase between 2 to 9 days (48 – 216 h) post-administration in comparison to time 0 as seen in Figure 3.4. This increase in the later hours is advantageous as it shows a pharmacological effect of TACA on the biomarker levels which indirectly extends the detection period of TACA from 2.5 to 9 days post-administration. Using ANOVA, the figures in this time period were consistent with pre-administration samples (FC < 1.5 and > 0.05).This would possibly also allow racing authorities to determine if a corticosteroid had been administered in line with their rules (AR 87) which state that a corticosteroid can only be administered intra-articularly at least 8 days (192 h) prior for the horse to run. An increase was also observed between 60 minutes and 6 hours that could also identify an administration close to race. Unfortunately, these two time points were also no significant to pre-administration samples. Due to the lack of placebo treated horses (controls) in the experimental design, it cannot be known if the OEA levels changing were due to TACA or from other external reasons. Whilst certain samples may not exhibit change compared to the individual horse itself (using pre-administration samples), the use of proposed PRLs for the biomarkers, whilst intelligence based may be useful to detect for the administration of a substance[113]. It must be noted that whilst the use of PRLs for biomarkers is advantageous, it cannot be conclusive of an administration, it is only indicative of an administration.

In comparison to the proposed PRL from chapter 2.4.2 for OEA, the upper PRL of 5.6 ng/mL, was exceeded by horse 1 and 3 at certain time points. For horse 1, time points of 4 and 5 days (96 – 120 h), whilst for horse 6, only the 5 days (120 h) post-administration time point exceeded the 5.6 ng/mL upper PRL. Comparatively, horse 5 at 8 h, 12 h and 1.5 days (36 h) whilst for horse 6, the time points of 12 h, 1 and 1.5 days (24 – 36 h) exceeded the lower PRL of 0.21 ng/mL. With TACA only detectable up to 2.5 days (60 h) post-administration, the exceedance of the upper PRL allowed for indirect detection up to 4 days (96 h) post-administration allowing for the potential for race day detection.

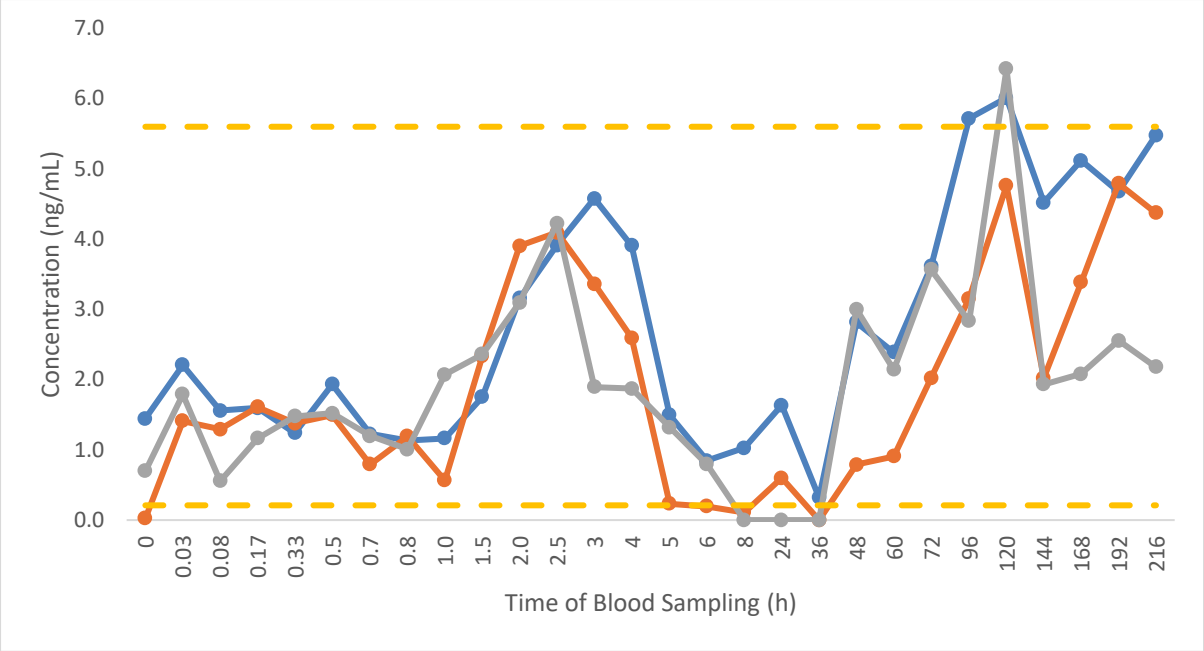

Figure 3.4: OEA concentrations for 3 horses (blue indicates horse 4, orange indicates horse 5 and grey indicates horse 6, yellow dotted line indicates the proposed upper (5.6 ng/mL) and lower (0.21 ng/mL) PRLs).

3.3.2.3 Corticosteroid Detection

From the 5 targeted corticosteroids of hydrocortisone (HC), cortisone (C), 18-oxocortisol, 18-hydroxycortisol and 11-deoxycortisol, only HC and C were detected, therefore the hydrocortisone to cortisone ratio (HC/C) could be applied to monitor the effects of TACA. A summary table containing the concentration obtained for the corticosteroids at each time point administered can be found in table A9 in the appendices. Concentrations of HC due to the accuracy having a high relative error in the method validation were deemed as estimations only. Estimated concentrations of HC can be seen in figure 3.5 whilst concentrations of C can be seen in figure 3.6.

The ratio of HC/C fell below the lower PRL of 0.12 as proposed in chapter 2.4.5. This was seen between 2.5 hours and 9 days (216 h) post-administration however between the three horses, a decreasing trend was not consistent as seen in Figure 3.7. This was specifically seen in the first 20 minutes for horses 5 and 6. The decrease of endogenous corticosteroids allowed for an indirect form of detection for TACA consistent with previous studies[93]. Decreased levels

were seen for varying lengths of time between the three horses. Levels were decreased in one horse for at least 9 days (216 h) post administration (horse 5 did not return to baseline by the end of the study). These time points compared to pre-administration samples had FC < 0.67 and a p-value of < 0.05 (for decreased biomarkers). For horse 4, a decrease was seen between 4 hours and 3 days (72 h) post-administration before returning to basal levels. For horse 5 however, there is no evidence of returning to basal levels following 2.5 hours post-administration whilst for horse 6 decreasing levels were seen from 2.5 hours to 8 days (192 h) post-administration. In comparison, HC/C ratio values were absent or decreased between 2.5 hours and 9 days (216 h) post-administration.

Using the upper PRL, only the 2- and 5-minute post-administration samples surpassed the upper PRL of 50.3 which unfortunately doesn't provide race-day detection. It must also be noted; this study was conducted on research horses which spend majority of their time in a non-stressful environment resulting in a lower HC compared to currently running racehorses. A study conducted by Soma *et al.* in 2011 investigated the administration of TACA and the effects on HC and C. From this study, the authors observed that following IA administration, HC had a significantly decreased concentration within 2 hours post-administration[108]. HC concentrations remained significantly different at 11 days post-administration[108]. Between the two studies, it is evident that TACA reduces the concentration of HC, however the time for individual horses to return to basal levels is different allowing for the possibility of individual reference limits from longitudinal profiling to provide indirect detection of corticosteroid administration.

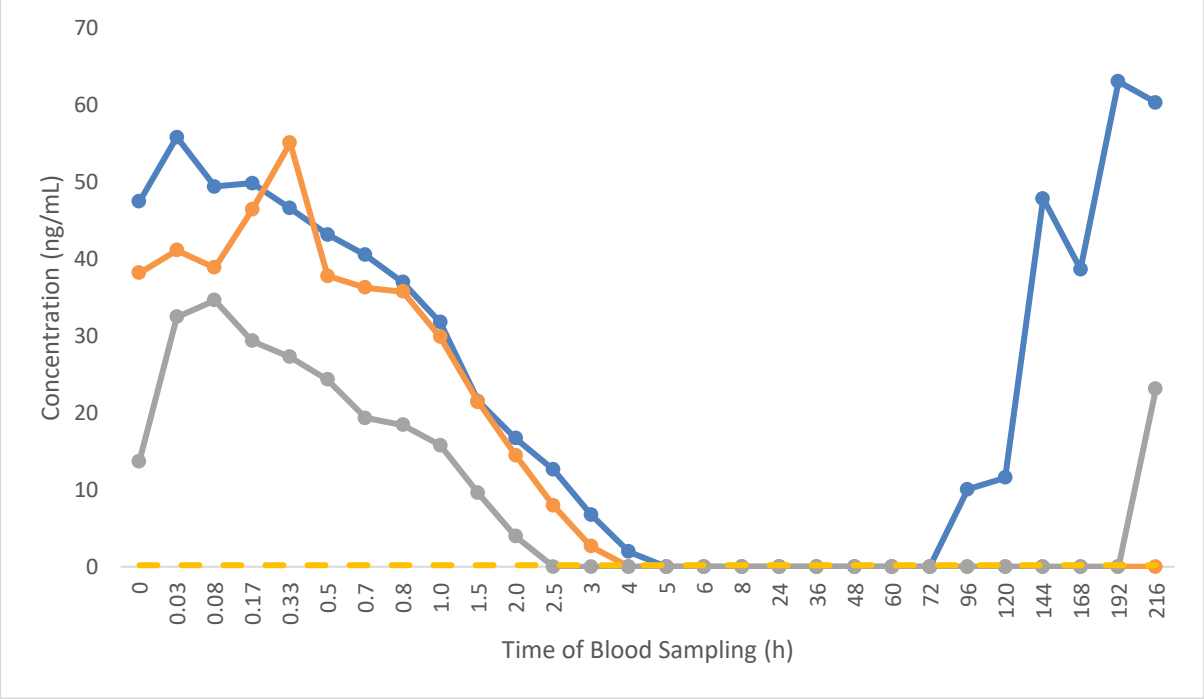

Figure 3.5: Estimated HC values for 3 horses (blue indicates horse 4, orange indicates horse 5 and grey indicates horse 6, yellow dotted line indicates the lower (0.2 ng/mL) PRL.

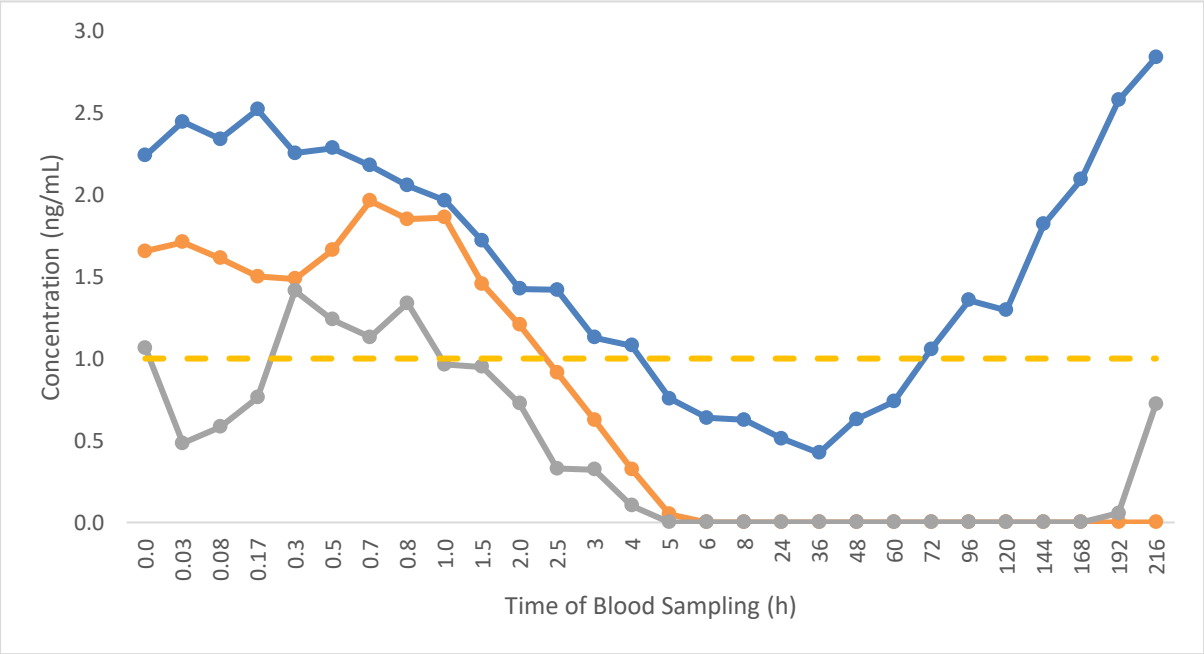

Figure 3.6: C values for 3 horses (blue indicates horse 4, orange indicates horse 5 and grey indicates horse 6, yellow dotted line indicates the lower (1.0 ng/mL) PRL.

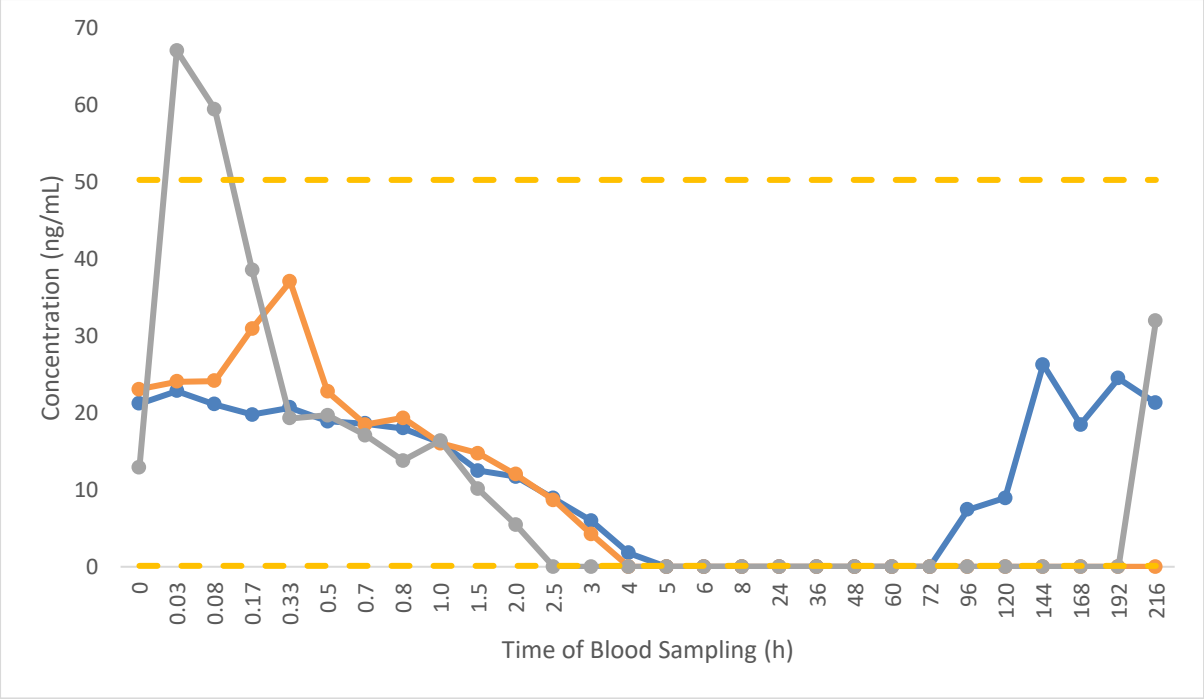

Figure 3.7: HC/C ratio values for 3 horses (blue indicates horse 4, orange indicates horse 5 and grey indicates horse 6, yellow dotted line indicates the upper (50.3) and lower (0.12) PRLs).

3.3.3 Semi-Targeted Detection

Following the semi-targeted screening data processing method, compounds that fit the set criteria (see chapter 2.2.8.2 Semi-Targeted Screening) had the peak area plotted. Compounds that showed change throughout the administration in comparison to time 0 were subject to further analysis. The compounds of 12-HETE, 13-HDHA, 15-HEDE, 17-HDHA, 18-HEPE and 9-HOTrE had concentrations estimated (with the corresponding internal standard) to determine if the estimated concentrations would provide similar pattern to the peak area response. A summary table containing either the concentration or peak area obtained for the semi-targeted compounds at each time point administered can be found in tables A10 to A12 in the appendices. Table 3.3 highlights the semi-targeted compounds and the change seen throughout the administration.

Table 3-3: Semi-targeted compound change throughout the administration

Compound	**Change Observed**	**Time points post administration**
13-HDHA	Decrease	7 – 9 days (168 – 216 h)
17-HDHA	Decrease	7 – 9 days (168 – 216 h)
15-HEDE	Decrease	3 – 9 days (72 – 216 h)
18-HEPE	Decrease	1.5 – 9 days (36 – 216 h)
9-HOTrE	Increase	3 hours – 1 day (24 h)
12-HETE	No distinct pattern but consistently detected	

Since 12-HETE provided consistent peak areas for the entire testing period, this showed the potential for 12-HETE being an endogenous reference compound. These 6 compounds underwent quantification (for estimated concentrations) in a similar manner to the targeted compounds with only 12-HETE and 9-HOTrE showing potential to be added as a targeted compound. Consistent estimated concentrations were seen with 12-HETE, and level increases were seen with 9-HOTrE at 3 hours post-administration for all three horses. Figure 3.8 highlights the estimated concentration plot for 12-HETE whilst Figure 3.9 shows the peak area plot for 9-HOTrE. Based on the high estimated concentrations, 12-HETE whilst having extremely variable concentrations has the potential to be an endogenous reference compound for any proposed ratios against another compound due to its consistent detection. There is an increase of 9-HOTrE between 3- and 4-hours post-administration, there is however evidence of returning to baseline compared to pre-administration levels between 7 to 9 days (168 – 216 h) post-administration. This return to baseline at the later time points could be advantageous as discussed in chapter 3.3.2.2 and 3.3.2.3 with the indirect detection via the lipids extending the detection window of TACA from 2.5 to 9 days (60 – 216 h) post-administration. Given that the estimated concentration is high (all concentrations for 9-HOTrE are estimates as it currently does not fall on the calibration curve), the monitoring of 9-HOTrE may have to come from the peak area or possibly a ratio between 9-HOTrE and 12-HETE. Further work from the other horses will be required for this conclusion to be made for other administrations.

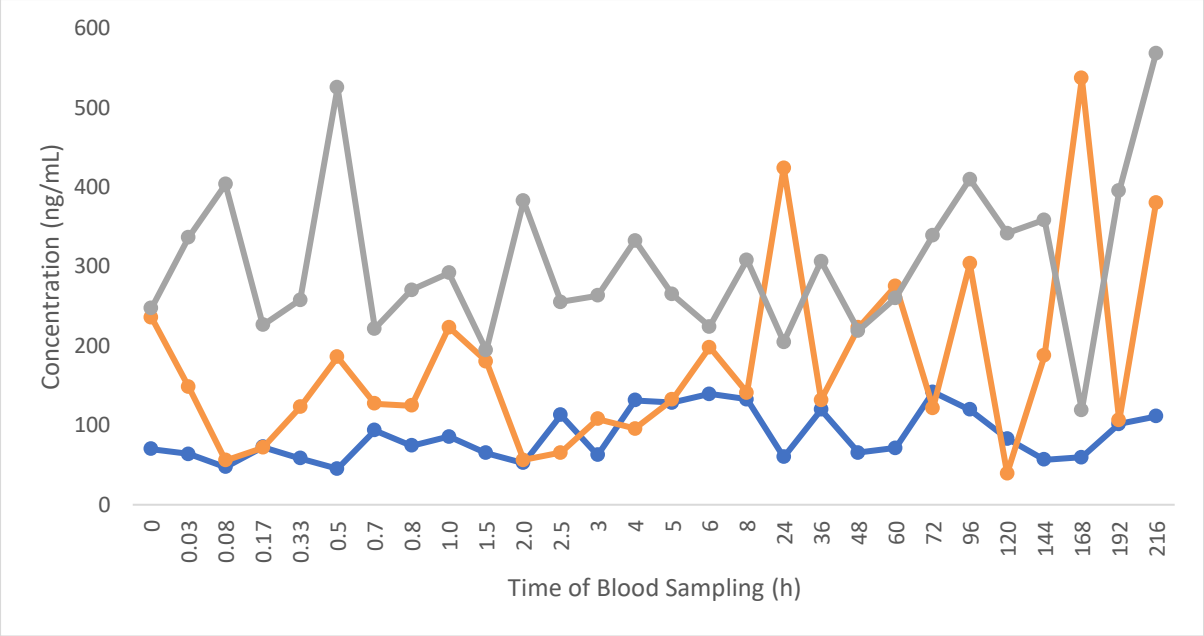

Figure 3.8: Estimated 12-HETE concentrations for 3 horses (blue indicates horse 4, orange indicates horse 5 and grey indicates horse 6)

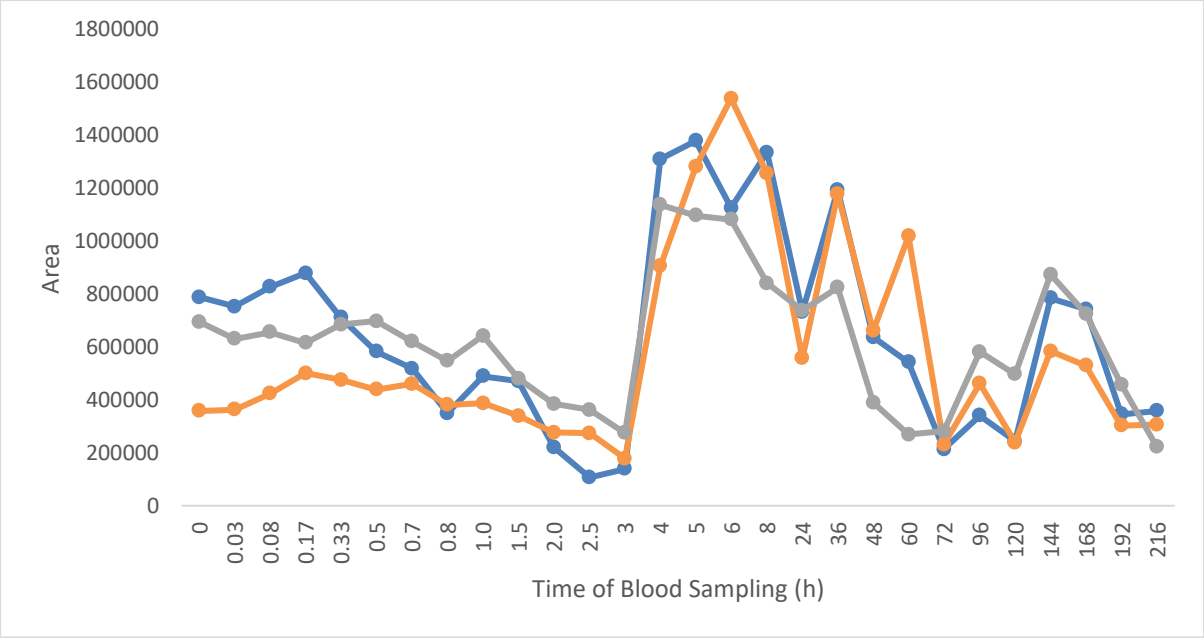

Figure 3.9: Peak area plotted for 9-HOTrE from initial run for 3 horses (blue indicates horse 4, orange indicates horse 5 and grey indicates horse 6).

3.3.4 Non-Targeted Detection

Three time points were chosen to undergo the non-targeted screen process (as outlined in chapter 2.2.8.3). These time points were chosen based on the maxima of TACA and any potential sampling time points that could mimic a routine analysis time. This was also completed in accordance with rule AR87, from this, the time points of 20 minutes, 4 hours and 8 days (192 h) were chosen for untargeted analysis. Untargeted analysis has currently only been conducted for horse 4.

Refer to Table 3.4 for the results generated from the untargeted workflow. Masses of 480.3081, 506.3235 and 573.2067 were determined to be of interest with 480.3081 (from heat map) and 506.3235 (from volcano plots) being primarily up-regulated and 573.2067 being primarily down-regulated from heat map.

Table 3-4: Results generated from the developed untargeted workflow.

Mass	Time Sampled	Proposed Formula
480.3078	20 minutes	$C_{22}H_{44}N_3O_7F$ ($COOH^-$)
480.3081	4 hours	$C_{21}H_{42}N_3O_5F$
480.3082	8 days (192 h)	$C_{25}H_{43}N_3O_6$ ($COOH^-$)
480.3083		$C_{24}H_{41}N_3O_4$
480.3084		
480.3087		
506.3239	20 minutes	$C_{27}H_{45}N_3O_6$ ($COOH^-$)
506.3235	4 hours	$C_{26}H_{43}N_3O_4$
506.3234		$C_{24}H_{46}N_3O_7F$ ($COOH^-$)
		$C_{23}H_{44}N_3O_5F$
573.2067	8 days (192 h)	$C_{40}H_{30}O_4$ ($COOH^-$)
		$C_{39}H_{28}O_2$

		$C_{31}H_{32}N_4O_4FP$ ($COOH^-$) $C_{30}H_4O_2FP$

Following a search using the Human Metabolome Database (HMDB) for the various masses and ChemSpider for the proposed formulas, unfortunately these searches did not predict appropriate results for the identification of these masses or proposed formulas. This can also be due to the limitations of the database used. It is also likely this is due to the incorrect proposed formula from the instrument or additional adducts causing incorrect masses to be generated. Further work will be necessary to determine if these masses can relate to an appropriate biomarker for expanding the scope available for the indirect detection of TACA. These can include further investigating the MS2 spectra to perform structural elucidation or performing searches on databases using the MS2 spectra to determine if it is consistent with another biomarker.

3.4 Equine Biological Passport – Individual Reference Limits

Unfortunately, due to the lack of pre-administration samples taken from the horses that were a part of this study, no profiles could be generated as there weren't enough samples to determine baseline values for each horse.

3.5 TACA Conclusion

From this pilot 3-horse study, it was concluded from the 100 µL method that TACA was detectable up to 2 days post-administration. With the targeted monitoring of lipid and corticosteroid biomarkers, many of these were consistently detected throughout the administration. This includes 15(S)-HETE, 5(S)-HETE, AA, PGD_2, $PGF_{2\alpha}$ and AEA for the lipids and HC and the HC/C ratio for the endogenous corticosteroids. The ability to detect TACA was indirectly extended up to 9 days (216 h) through the pharmacological effects of TACA on the biomarker levels being monitored. Therefore, the use of these biomarkers would allow for intelligence that a potential administration of an intra-articular corticosteroid administration was used however, further confirmation of the exogenous drug would be necessary. Semi-targeted detection has allowed for the incorporation of 12-HETE, 13-HDHA, 15-HEDE, 17-

HDHA, 18-HEPE and 9-HOTrE into the targeted screen for future exogenous administrations. Unfortunately, from the untargeted detection, no new novel compounds could be identified. As this study only utilised 3 horses, power calculations or sample size analysis were not conducted. Statistical analysis would be more significant when a larger number of horses were to be studied under this administration in the future. Further work would be required to observe whether other horses' samples administered with TACA using the same dose and joint would produce similar results.

TACA References

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Chapter 4: Fluticasone Propionate Administration

4.1 Introduction

The use of corticosteroids in the racing industry can treat inflammation in the horse with treatment usually administered intra-articularly (e.g. TACA[62,109]) as it is specifically targeted to the affected joints. However, there has been an increase in the use of inhaled administrations for corticosteroids especially to help with inflammation. Fluticasone propionate (FLP) is a topical corticosteroid that is commonly used as an anti-asthmatic agent in humans[114]. FLP can be administered in a nebulised form through a dose inhaler coupled to a spacer device[114]. This allows the entire dosage to be inhaled appropriately. With this, FLP is used to treat respiratory disorders in horses, for example Inflammatory Airway Disease[114].

The major challenge regarding the targeted detection of an inhaled substance is the likelihood of the concentrations being extremely low in plasma and urine[114]. This is not only for the parent compound itself but also for the major metabolites such as the carboxylic acid from FLP[114]. Although FLP is usually administered in relatively high concentrations compared to that of an intra-articular, intra-muscular or intra-venous injection, the deposition in the lungs and further distribution within the equine system can be low[114]. In humans, the understanding of FLP pharmacokinetics is reasonably known[114] however, in the horse, research is relatively scarce. In human studies, participants were able to inhale the dose as instructed, however in the horse, this was difficult to replicate[114]. For full effect from FLP, the treatment should be directly applied to the lower respiratory tract[115].

In a study conducted by Gray *et al.* the authors developed a method for the extraction and detection of FLP in horse plasma and the relevant metabolite in urine[114]. The authors utilised a triple quadruple mass spectrometer coupled to an ultra-high performance liquid chromatograph for the analysis[114]. This method was used to analyse plasma and urine samples collected following an inhaled administration from six horses. From this study, the parent compound FLP was detectable in plasma samples for a minimum of 3 days post-administration with two horses showing detection of FLP for the entire duration of the study (104 hours)[114]. Concentrations however, were low at less than 10 pg/mL with urine metabolite detection lasting until 18 hours post-administration[114].

4.1.1 Aims

The aim for this study was to determine if FLP was detectable using 100 µL of equine plasma for complementary biomarker analysis since the method utilised by Gray *et al.* had 2 mL of sample volume. In addition to the direct detection of FLP, the secondary aim was to determine whether lipid or corticosteroid biomarkers could indirectly detect for FLP administration. As FLP is administered via inhalation, AR87 does not apply in this situation, therefore, according to the rules of racing, FLP must not be detected for a horse presented to race (i.e. on race day).

4.2 Materials and Methods

4.2.1 Chemicals and regents

Refer to chapter 2.2.1.

4.2.2 Reference Materials

Refer to chapter 2.2.2 for the lipid and corticosteroid biomarkers.

FLP obtained for calibration and quality control was manufactured by Toronto Research Chemicals (Toronto, Ontario, Canada) and purchased from PM separations (Capalaba, QLD, Australia). The corresponding internal standard FLP-D_5 was manufactured and purchased from Sigma Aldrich – Supelco (St. Louis, Missouri, United States of America).

4.2.3 Sample preparation for FLP, lipid and corticosteroid biomarkers

Refer to chapter 2.2.5 for the extraction of the lipid and corticosteroid biomarkers. This method was also used for the extraction of FLP.

4.2.4 FLP administration study

A 3-horse study (Horse 7, 8 and 9) was completed via inhalation using a metered dose inhaler to the nasal passage of the horse. A dose of 2 mg was administered to each horse at a rate of 250 µg/metered dose. Baseline samples were collected from 7 days prior administration to 1 day prior to the administration of FLP. Samples were then collected at 2, 5, 10, 20, 30, 40, 50, 60 and 90 minutes then 2, 2.5, 3, 4, 6, 8, 12 hours, 1, 2, 3, 4-, 5-, 6- and 7-days post-administration. Blood samples were immediately centrifuged at 1,500 x *g* for 10 minutes to obtain the plasma after collection and stored at -20 °C until analysis. Samples from this administration underwent one freeze thaw cycle prior to extraction and analysis. Animal ethics approval (A19382) was obtained for this study from Charles Sturt University Animal Care and Ethics Committee and reviewed by the Racing NSW Animal Care and Ethics Committee.

4.2.5 Instrument conditions

Please refer to chapter 2.2.6 instrument parameters

4.2.6 Method validation preparations

Please refer to chapter 2.2.7 for the method validation parameters assessed. The method validation requirements include linearity, sensitivity, accuracy, precision, recovery, matrix effects and stability as outlined by Peters *et al.*[99].

4.2.7 Data Analysis Parameters

All data acquired using the LC-HRMS instrument was processed using the Shimadzu Insight Explore software (Version: 3.8 SP1) with further data processing completed on Excel (version 16.71) and MetaboAnalyst (version 5.0). For further information on the raw data, refer to chapter 2.2.8.1. For data processing methods, refer to chapter 2.2.8.3 Semi-Targeted Screening data processing and 2.2.8.4 Non-Targeted Screening Data Processing.

4.3 Result and Discussion for an FLP administration

4.3.1 Method Validation for FLP

Method validation was performed with assessment of linearity, sensitivity, accuracy, precision, recovery, matrix effects and stability using the concentration of FLP determined relative to D_5-FLP.

4.3.1.1 Linearity

Calibrators for FLP were prepared at 0 ng/mL, 0.05 ng/mL, 0.1 ng/mL, 0.2 ng/mL, 0.5 ng/mL, 1 ng/mL, 2 ng/mL, and 5 ng/mL. The averaged R^2 value across 3 batches of calibration curves was determined to be 0.9983 indicating a linear calibration curve as seen in Figure 4.1 . Residual analysis (n=3) showed no bias in the data through the random placements of the residual data points for each concentration as seen in Figure 4.2.

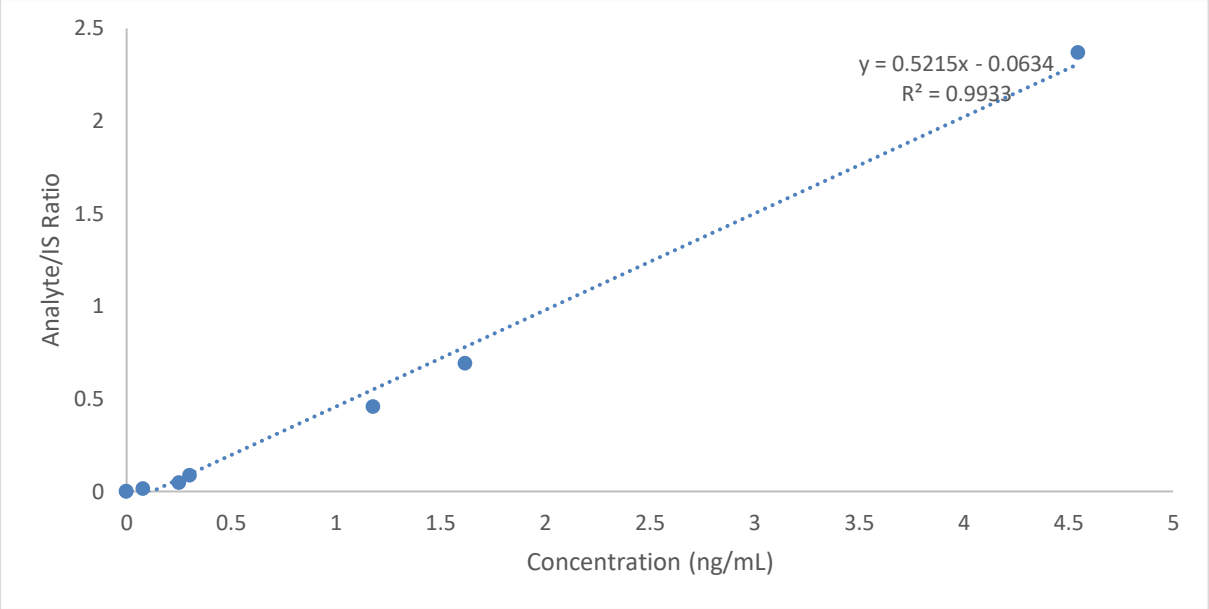

Figure 4.1: Averaged linearity of FLP (n=3)

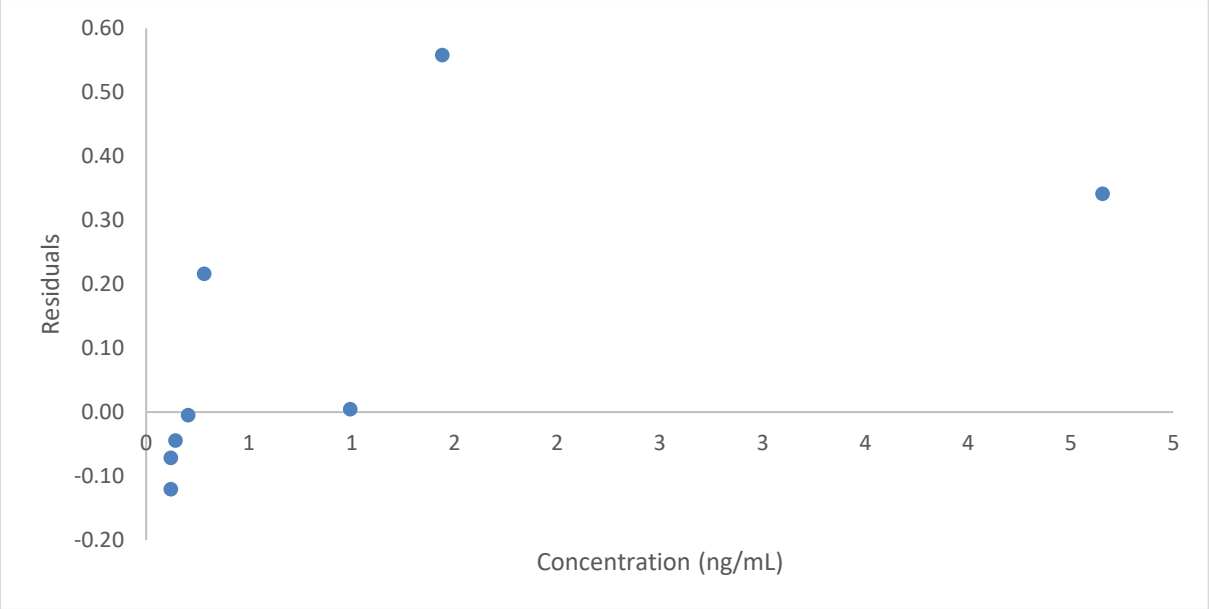

Figure 4.2: Residual analysis of linearity assessment for FLP (n=3)

4.3.1.2 Sensitivity

As FLP was only acquired in positive ESI mode, one set of LOD and LOQs are required for FLP. Similar to TACA, the LOD and LOQ were estimated by visual inspection of peaks with *S/N* of 3 or greater for LOD and *S/N* of 10 or greater for LOQ. For FLP, the estimated LOD was 0.1 ng/mL whilst the estimated LOQ was 0.2 ng/mL.

4.3.1.3 Accuracy, Precision, Recovery, Matrix Effects and Stability

Accuracy and precision were assessed as relative error (% RE) and relative standard deviation (% RSD), respectively at three QC levels in replicates of 7. QC levels chosen for FLP were 0.5 ng/mL for low QC, 1 ng/mL as medium QC and 2 ng/mL as high QC. Precision and recovery were within acceptable ranges for equine plasma being less than 15% RSD for precision and above 60% for recovery with the exception of the HQC for precision. The precision at 2 ng/mL is slightly higher than 15% which could be due to the known sensitivity issues related to the LC-HRMS and the volume of sample used (100 µL). Matrix effects however, showed large ion enhancement for both LQC and HQC being above 100%[102] whilst for the MQC, there is evidence of ion suppression due to being under 100%[102]. Accuracy, however, was higher than the acceptable relative error of 25% for all three QCs levels therefore there is limited

confidence in the accuracy of FLP concentrations. As noted previously, this could be due to the small interfering chromatographic peaks from the sample matrix. Considering the trend observed for decreasing concentrations, the reason for this may be the low signal intensity obtained for low concentrations in conjunction with small sample volume used for the biomarker method. For stability, FLP was deemed stable at 4 °C and -20 °C for up to 2 months. Table 4.1 highlights the accuracy, precision, recovery, and matrix effects results for FLP.

Table 4-1: Accuracy, precision, recovery, and matrix effects for FLP (n=7)

Quality Control Level	Accuracy (% RE)	Precision (% RSD)	Recovery (%)	Matrix Effects (%)
LQC (0.5 ng/mL)	88	8.1	88	232
MQC (1 ng/mL)	52	14	69	91
HQC (2 ng/mL)	48	19	74	182

4.3.2 Targeted Detection

4.3.2.1 FLP Elimination

The administration of FLP was completed with quantification against D_5-FLP as the corresponding internal standard. A total of 3 horses had samples extracted and analysed for a period from 7 days prior to administration up to 7 days post-administration. Unfortunately for all three horses, the 100 µL method could not detect FLP throughout the entire administration. This could be due to sensitivity limitations of the biomarker method and/or the route of administration not absorbing the inhaled FLP in comparison to a direct injection. With inhalation, whilst FLP was administered through a meter, it is unknown how much of the FLP was completely inhaled and absorbed through the airways of the horse. With that, FLP must then be absorbed into the blood stream to be detected in equine plasma. As it was not detectable, this poses the question of how much was firstly inhaled by the horse and secondly, how much if any was absorbed into the equine system. Another reason for possible non-detection is whether the detection is of the correct substance. Whilst the method is

validated for FLP, FLP is the propionate ester of fluticasone. It is unknown whether fluticasone, following hydrolysis of the ester by plasma esterase, and not FLP is present in the plasma. Finally, the lack of detection could have resulted from the small amount of sample (100 µL) extracted. If this extraction was completed in a routine application, a larger amount of sample would be used.

Therefore, for the detection of FLP using this 100 µL sample method, it was crucial to detect a pharmacological effect from FLP using the lipid and corticosteroid biomarkers to have the ability to indirectly detect inhaled FLP.

4.3.2.2 Lipid Detection

Of the 19 target lipids, 7 were detected in all 3 horses, 15(S)-HETE, AEA, 5(S)-HETE, cortisone, 18-HEPE, OEA and HC (For raw data, refer to appendices tables A13 to A16). The majority of the lipids detected remained consistent throughout the study period with no increase or decrease of concentrations in comparison to time 0. These biomarkers included 15(S)-HETE, 5(S)-HETE, the 5(S)-HETE to 15(S)-HETE ratio, AEA, cortisone and 18-HEPE. It must be noted that all concentrations (with the exception of OEA and C) are deemed as estimations only.

One of the lipids that showed some change in the later time points was OEA as seen in Figure 4.3 (for OEA data and pre-administration data sampling, refer to appendices table A15 and figure A5). It must be noted that a limitation to this study is the lack of placebo treated horses (controls) in the experimental design. It cannot be known if the OEA levels changing were due to the effects of FLP or from other external reasons. An increase was seen between 2 to 4 hours post-administration before a decrease at 6 hours. This represented a percentage change of 48% in comparison to time 0, however, these time points were consistent with pre-administration samples (FC < 1.5 and p-value > 0.05). From 8 hours to 7 days (168 h) post-administration, a general increasing trend was observed with the largest percentage change at 3 days (72 h) post-administration showing an increase of 623% in comparison to time 0. There was also an increase observed at 7 days (216 h) post-administration where an increase of 293% in comparison to time 0 was seen. The time points between 3 h and 4 days (96 h) post-administration in addition to 7 days (216 h) post-administration displayed most change with fold changes greater than 1.5 and p-values of less than 0.05 in comparison to pre-

administration samples. However, the time points of 5 and 6 days (120 – 144 h) were consistent with pre-administration samples. This consistent increase, particularly in the later hours of the administration is advantageous as it can indirectly extend the detection period, potentially enabling race-day control of inhaled FLP. This supports the rules of racing stating that any legitimate therapeutic medication must be administered at least one clear day prior to race.

Implementing the proposed upper and lower PRLs for OEA from chapter 2.4.2, it can be seen that only horse 6 (grey) would have samples deemed abnormal. The sample at 3 days (72 h) post-administration with an OEA concentration of 10 ng/mL surpassing the upper PRL of 5.6 ng/mL would be deemed abnormal. The sample at 4 days (96 h) was calculated to be 4.85 ng/mL just falling under this threshold.

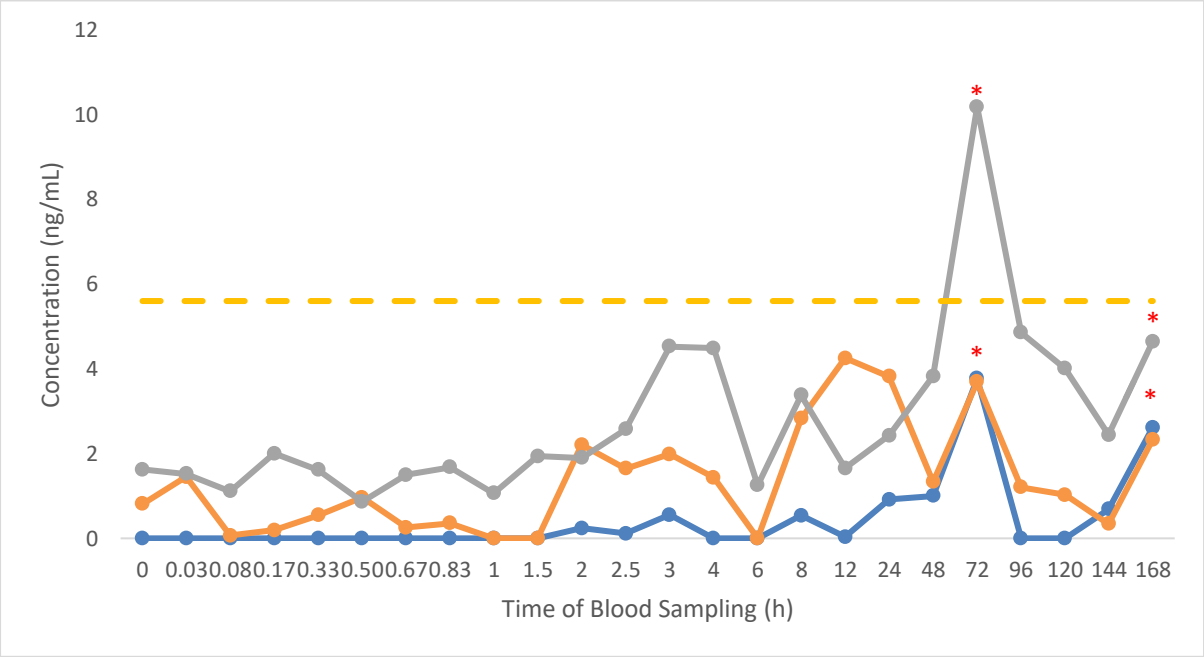

Figure 4.3: OEA concentrations for 3 horses with the yellow dotted lines indicating the proposed upper threshold for OEA (5.6 ng/mL) and red asterisks indicate significantly different time points compared to pre-administration samples (blue indicates horse 7, orange indicates horse 8 and grey indicates horse 9).

4.3.2.3 Corticosteroid Detection

Of the 5 target corticosteroids monitored (HC, C, 18-oxocortisol, 18-hydroxycortisol and 11-deoxycortisol), only HC and C were detected, therefore the biomarkers of HC and HC/C were investigated to determine the effects of FLP. It must be noted that all concentrations (with the exception of C and HC/C) are deemed as estimations only. Firstly, monitoring the effects on HC concentrations is presented in Figure 4.4 with data for HC and C in appendices table A16 (for pre-administration sampling, refer to appendices figure A6 for HC and A7 for HC/C).

Table 4-2: Statistics for HC for Horse 7 and Horse 9 during an FLP administration compared to pre-administration samples

	Horse 7			**Horse 9**		
Time point	24 h (1 day)	48 h (2 days)	72 h (3 days)	24 h (1 day)	48 h (2 days)	72 h (3 days)
% Change	-71.0	2.60	91.8	-13.5	351	576
Fold Change	-0.710	0.0260	0.0004	-0.135	3.52	5.76
p-value	0.05	0.94	0.02	0.88	0.006	0.0004

Table 4-3: Statistics for HC for Horse 8 during an FLP administration compared to pre-administration samples

	Horse 8			
Time point	96 h (4 days)	120 h (5 days)	144 h (6 days)	168 h (7 days)
% Change	1004	1534	1406	1081
Fold Change	10.0	15.3	14.1	10.8
p-value	0.000980	7.27×10^{-5}	0.000130	0.000630

According to Table 4.2, there was an increase between 1 to 3 days (24 – 72 h) post-administration for horses 7 and 9. Only the time points of 1 day (24 h) for horse 7 and 2 and 3 days (48 – 72 h) for horse 9 were showed considerable change compared to pre-administration samples. Comparatively for horse 8, an increase (170%) was observed from 3 to 5 days (72 – 120 h) post-administration. All three horses then showed decrease in HC with horse 8 showing a more pronounced trend. For horse 8 (as seen in Table 4.3), all samples from 4 to 7 days (96 – 168 h) post-administration had noticeable change to pre-administration samples (FC > 1.5 and p-values < 0.05). For horse 7, HC returned to basal levels at 6 days (144 h) post administration with no samples after this time point significantly different in comparison to pre-administration samples. For horse 8 and 9, concentrations remained increased in comparison to time 0. There is evidence of basal levels being approached (both horses at 7 days (168 h) post-administration had noticeable change compared to pre-administration samples with fold change and p-values at 10.8 and 0.000630 for horse 8, 3.8 and 0.00461 for horse 9). However, as samples were not collected after 7 days post-administration, it is unknown when basal concentrations were achieved.

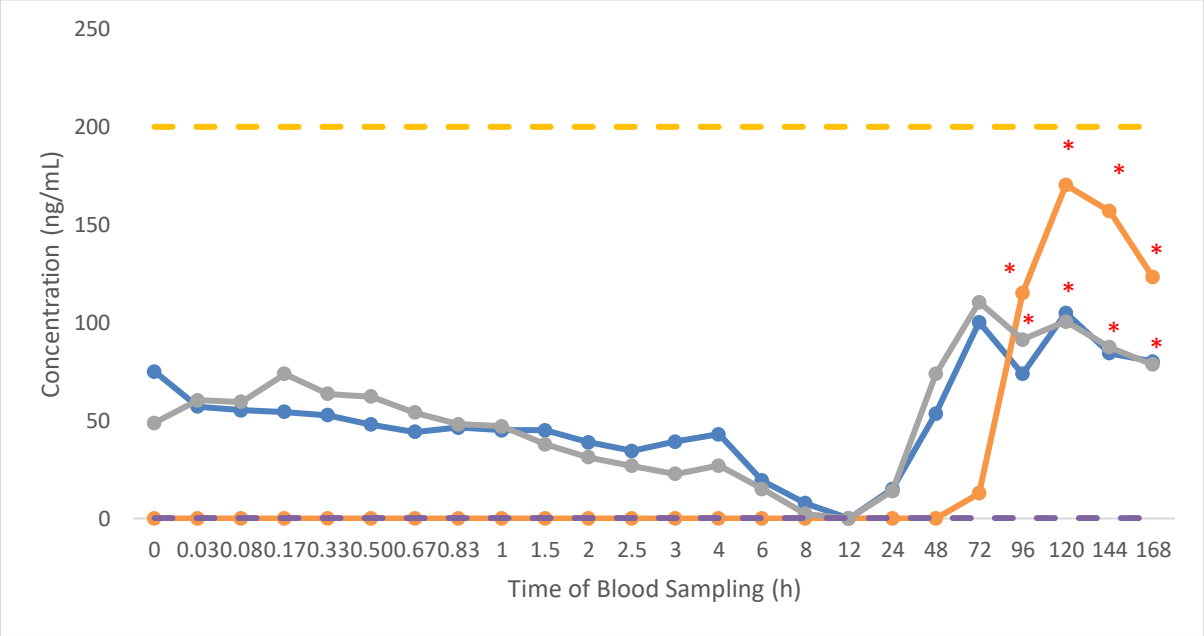

Figure 4.4: Estimated HC concentrations throughout an FLP administration (blue indicates horse 7, orange indicates horse 8 and grey indicates horse 9, yellow dotted lines represent the proposed upper (200 ng/mL) and lower PRL (0.2 ng/mL)) and red asterisks indicate significantly different time points compared to pre-administration samples.

With cortisone remaining consistent but detectable not exceeding 3.5 ng/mL (C data, refer to appendices table A16), the HC/C ratio was applied to monitor the effects of FLP. As seen in Figure 4.5, for HC/C values (for pre-administration data, refer to appendices figure A7) for each horse displayed inter-individual variation. However, as a commonality, all three horses showed a decrease of HC/C from 6 hours to 1 day (24 h) post-administration with the minima at 12 hours post-administration for all three horses being below the proposed PRL of 0.24[93]. Several samples that saw noticeable change are presented in table 4.4.

Table 4-4: Statistics for HC/C for Horses 7,8 and 9 during an FLP administration

	Horse 7		**Horse 8**			**Horse 9**
Time point	8 h	12 h	96 h (4 days)	120 h (5 days)	144 h (6 days)	72 h (3 days)
% Change	-67.6	-100	614	463	420	172
Fold Change	-0.676	-1.00	6.14	4.63	4.20	1.71
p-value	0.00506	0.00057	0.00842	0.0291	0.0423	0.0500

Samples that had noticeable change were predominately between 6 hours to 6 days (144 h) post-administration as seen in Table 4.4. During the time periods of 6 hours to 1-day (24 h) post-administration, this would have the potential for race day control as it is within the rules of racing stating that legitimate therapeutics must only be administered at least one clear day prior to race. With the additional time periods of 6- and 8-hours post-administration being decreased, this provides an additional 6 hours of indirect detection. This highlights the advantages of utilising biomarkers compared to the direct detection of a compound. Whilst the use of biomarkers is advantageous, this will always be a complementary technique with any confirmation of substances requiring a direct detection of the substance itself.

This was the presence of an increase for all three horses with horse 8 exceeding the proposed upper PRL for HC/C (58) at 3 days post-administration. This provided an extended indirect detection following FLP administration with the potential of controlling misuse close to race. Horses 7 and 9 do not exceed the upper PRL but there was evidence of an increase indicating

the positive pharmacological feedback of the equine system after the decrease in ratio value. Horses 7 and 9 appear to return to basal level concentrations at approximately 4 days (96 h) post-administration, however for horse 8, HC/C values remain increased at the end of the blood sampling (refer to appendices figure A7 for full sampling data).

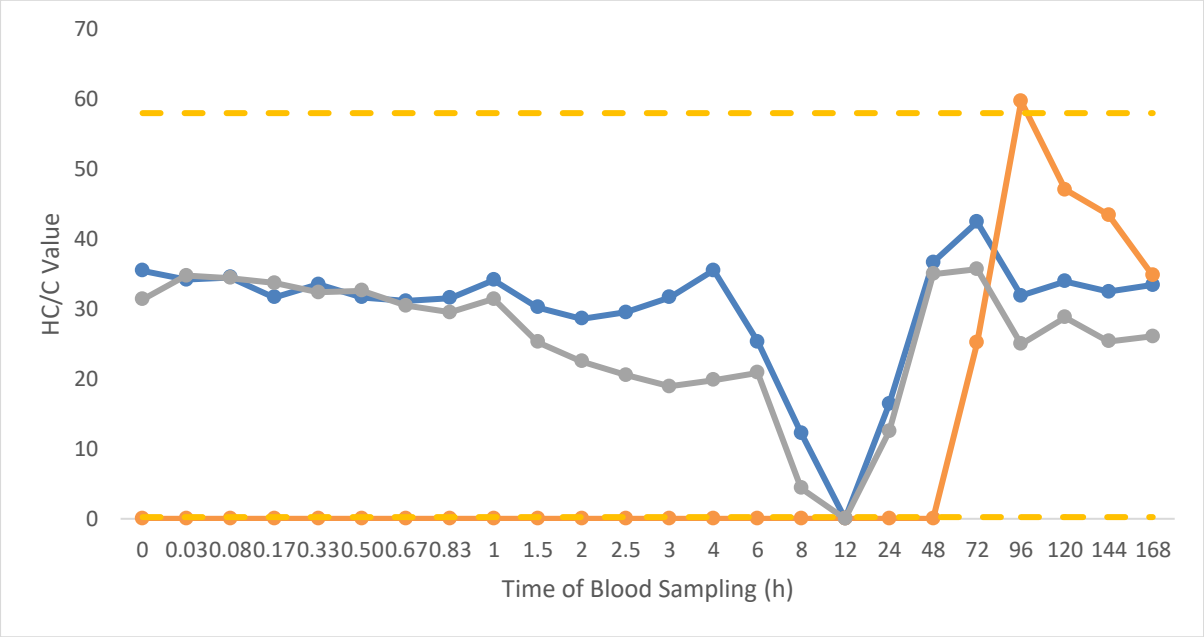

Figure 4.5: HC/C ratio values throughout an FLP administration (blue indicates horse 7, orange indicates horse 8 and grey indicates horse 9, proposed upper PRL (58) and lower PRL (0.24) in yellow dotted lines).

Between the monitoring of HC and C, it was evident that under the influence of FLP, the concentration of HC and HC/C values are decreased. Given how each horse showed different timing of effect and the return to basal levels, this allows the possibility of utilising individual reference limits from longitudinal profiling to show pharmacological effect providing indirect detection of an inhaled corticosteroid administration.

4.3.3 Semi-Targeted Detection

Following the semi-targeted screening data processing method (chapter 2.2.8.2), compounds that fit the set criteria would have the peak area plotted to determine if there was evidence of change for further analysis. The compounds 11-HEDE, 13-HODE, 14,15-DiHETE, 15-HEPE, 4-HDHA, 5-HEPE, 5-KETE, 9-HODE, DHA, and EPA provided the necessary information from

the peak area. Table 4.5 highlights the semi-targeted compounds and the change seen throughout the study period.

Table 4-5: Semi-targeted compound change throughout the administration

Compound	**Change observed**	**Time points post-administration**
11-HEDE	Increase	60 min – 4 hours
13-HODE	Increase	60 min – 4 hours
14,15-DiHETE	Increase	60 min – 4 hours
15-HEPE	Increase	60 min – 4 hours
4-HDHA	Decrease	3 – 7 days (72 – 168 h)
5-HEPE	Stable throughout study period	
5-KETE	Increase	60 min – 4 hours
9-HODE	Increase	60 min – 4 hours
DHA	Decrease	4 – 5 days (96 – 120 h)
EPA	Stable throughout study period	

From the semi-targeted compounds, 5-HEPE and EPA showed consistent peak areas for the study period providing potential to become an endogenous reference compound in the future. It was evident that 11-HEDE, 13-HODE, 14,15-DiHETE, 15-HEPE, 5-KETE and 9-HODE showed some change between 60 minutes and 4 hours which unfortunately doesn't allow for the potential of race day control. Comparatively, 4-HDHA and DHA displayed extended days of detection. Figure 4.6 highlights the integrated peak area for 4-HDHA using an internal standard already being analysed for the targeted method (5(S)-HETE-D_8) whilst Figure 4.7 shows the integrated peak area for DHA to a relevant internal standard (D_8-AA) that was already being analysed for the targeted method.

In Figure 4.6, 4-HDHA showed some decrease in the peak area in comparison to time 0. For horse 4 and horse 5, the decrease of 62% was observed at 4 days (96 h) post-administration before an increase between 5 to 6 days (120 – 144 h) post-administration by 21% and 36% respectively. At 7 days (168 h) post-administration, the integrated peak was decreased by 40% in comparison to time 0. Similarly for horse 8, a decrease of 26% was observed at 3 days (72 h) post-administration before being increased at 5 days (120 h) post-administration by 28%. 4-HDHA then proceeded to once again be decreased between 6- and 7-days (144 – 168 h) post-administration by 24% and 50% respectively. Horse 9 however, showed different results with decrease by 40% and 24% at 2- and 3-days (48 – 72 h) post-administration respectively. This was then increased by 33% in comparison to time 0 at 4 days (96 h) post-administration. The integrated peak area then returned to pre-administration levels between 5- and 6-days (120 – 144 h) post-administration before an increase by 27% at 7 days (168 h) post-administration. This inconsistent increase and decrease of peak area levels between the three horses doesn't allow for 4-HDHA to be a conclusive biomarker for the administration of FLP, however any deviations observed from longitudinal profiling could provide intelligence for further investigation.

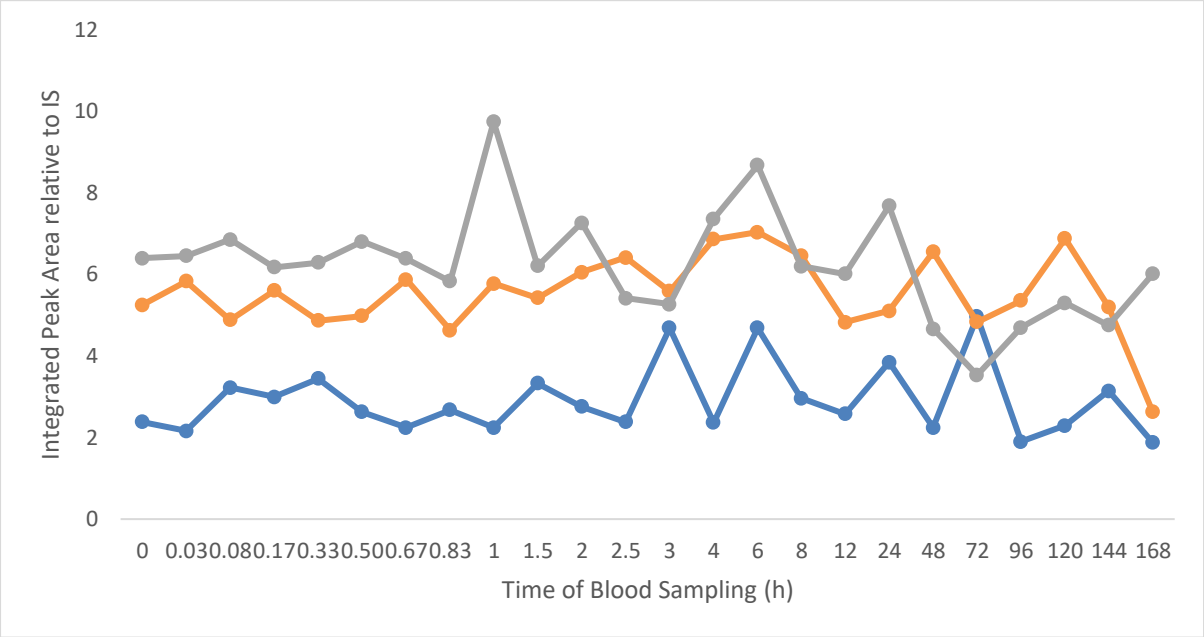

Figure 4.6: Integrated peak area of 4-HDHA for 3 horses during an FLP administration (blue indicates horse 7, orange indicates horse 8 and grey indicates horse 9).

Docosahexaenoic acid (DHA) similar to AA, is the precursor to another chain of lipids in a separate cascade, therefore it was hypothesised that the presence of this compound was expected in high amounts. In Figure 4.7, similar to 4-HDHA, DHA did not show consistent results between each horse. Horse 7 showed the most distinct change with a 106% increase at 2.5 hours post-administration, however for race day control, at 1-day (24 h) post-administration there was a 52% increase in comparison to time 0. DHA then showed a decrease of 64% at 4 days post-administration before increasing again between 5- to 6-days (120 – 144 h) post-administration by 63% and 55% respectively. Horse 8 showed a similar pattern with T-max at 2.5 hours post-administration by an increase of 24% in comparison to time 0. This was followed by a decrease to 4 days (96 h) post-administration by 36% before returning to pre-administration levels at 5 days (120 h) post-administration. In comparison, horse 9 showed a similar trend with T-max also at 2.5 hours post-administration with a 23% increase in comparison to time 0. Levels of DHA in horse 9 remained consistent with no distinct increasing or decreasing until 4 days (96 h) post-administration where a sharp decrease of 39% was observed before returning to pre-administration levels between 5- and 7-days (120 – 168 h) post-administration. In a similar manner to 4-HDHA, the presence of this biomarker surpassing basal levels can provide an indication of an FLP administration.

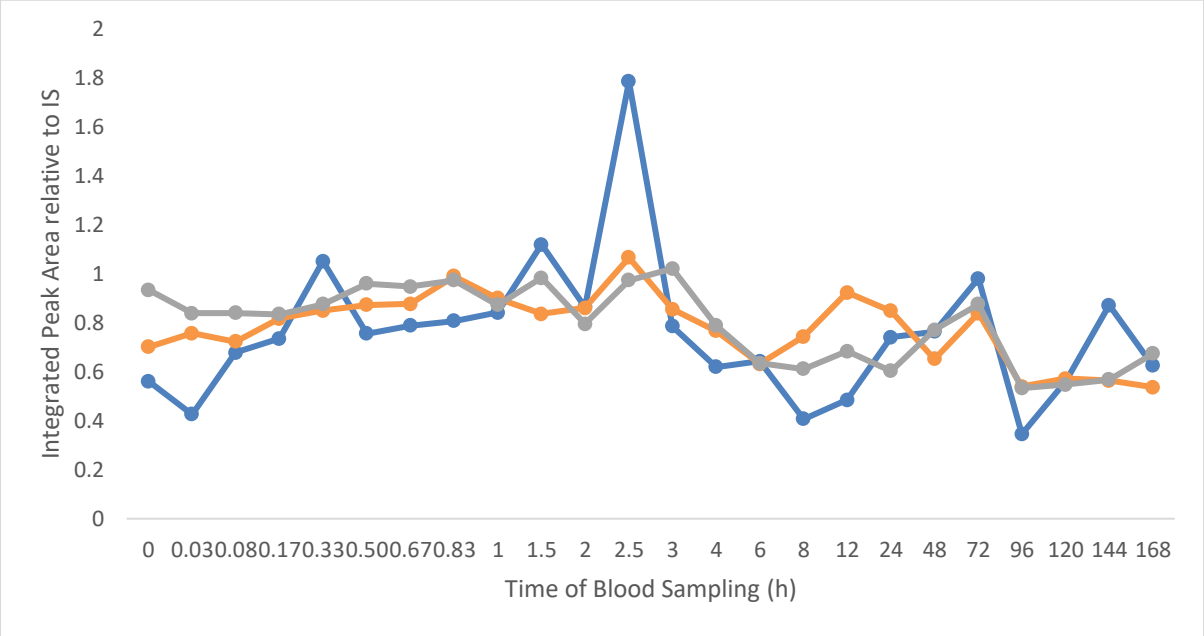

Figure 4.7: Integrated peak area of DHA for 3 horses during an FLP administration (blue indicates horse 7, orange indicates horse 8 and grey indicates horse 9).

4.4 Equine Biological Passport – Individual Reference Limits

As the administration of FLP included 7 pre-administration samples, this allowed for the use of individual reference limits to profile the HC/C ratio. The profiles are calculated using the equations as stated in chapter 2.2.10. Figure 4.8 highlights an example profile of HC/C using horse 7 in the presence of FLP.

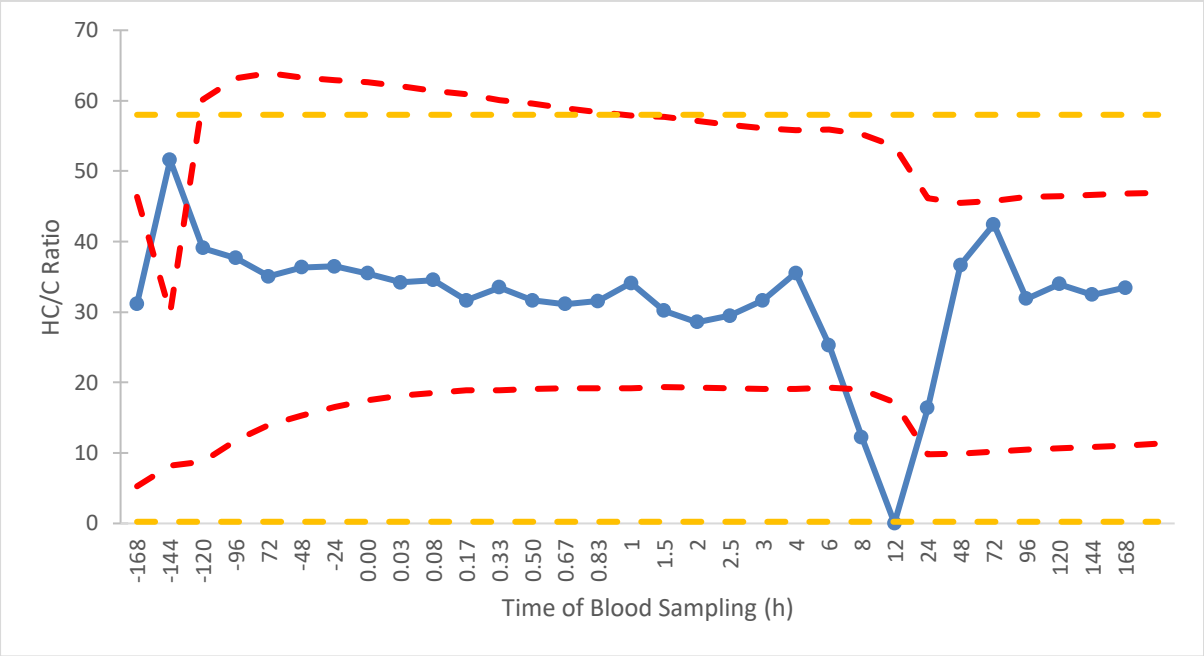

Figure 4.8: Intra-individual profile for the FLP administration for horse 7 using the HC/C ratio (Legend: blue = HC/C ratio, red = individual upper and lower thresholds, yellow = proposed population upper (58) and lower thresholds (0.24))

As seen in Figure 4.8, the HC/C ratio showed greater variance with one point that surpasses the lower PRL of 0.24 at 12 hours post-administration. If this sample was detected during routine screening, this would be an abnormal value allowing for potential investigation of an administration of a corticosteroid. With the addition of the IRLs, the point at 12 hours exceeds the PRL with an additional intelligence point at 8 hours post-administration where the point exceeds the lower IRL but not the PRL. At 1-day (24 h) post-administration, HC/C levels return to within the PRL and IRLs of this individual horse. This profile highlights the advantages of using IRLs in comparison to PRLs, but this should not deter the use of PRLs as the more traditional form of doping control. The use of IRLs and longitudinal profiling is not a form of

confirmation as it is only an intelligence model, therefore any sanctions will require the use of traditional confirmations as necessary.

For the profiles of the other 2 horses (Horse 8 and Horse 9) that underwent an FLP administration, profiles can be found in appendix Figures A8 and A9.

4.5 FLP Conclusions

The use of corticosteroids in the racing industry to treat inflammation can be by injection, intra-articular or intra-muscular. However, the increased use of inhaled corticosteroids for improved respiratory efficacy has the potential to complicate analytical detection due to the low concentrations of parent compounds. Therefore, the use of biomarkers can be considered for indirect detection.

From this 3-horse pilot administration study, it was observed that using 100 µL of equine plasma, FLP could not be detected at a quantifiable level. From the targeted detection of 19 endogenous lipids and 5 corticosteroids, OEA, HC and HC/C values detected pharmacological effects which were able to provide indirect race-day detection of a potential FLP administration. Additionally, the use of IRLs showed the potential for the EBP to detect the pharmacological effect of an FLP administration in addition to the proposed PRL. From the semi-targeted method, the two compounds 4-HDHA and DHA showed effect under the influence of FLP, however there the peak areas monitored showed inconsistent levels. Nonetheless, the measurement of OEA, HC (estimated) and HC/C values has showed the potential for indirect detection of FLP which could be beneficial in the absence of the parent compound. Further work is required to observe whether a large sample size (i.e. 10 horses) under an administration of FLP using the same dosage would produce similar result and conclusions.

FLP References

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Chapter 5: Bisphosphonate Administrations

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Lipid and corticosteroid biomarkers under the influence of bisphosphonates (DOI: 10.1002/dta.3811)[116]

5.1 Introduction

The bones within a race horse have a solid but dynamic structure that undergoes a consistent remodelling process that is primarily controlled by osteoblasts and osteoclasts in addition to any tissue or nerves[117]. Osteoclasts break down bone whilst osteoblasts replace any removed bone over a period of time in which it also mineralises[117]. Bisphosphonates (BPs) were first synthesised in the 1800s however, it wasn't until the 1960s when BPs were first introduced to treat bone disorders affecting osteoclasts[118]. BPs are large hydrophilic molecules that don't easily bypass the lipid membrane, are poorly absorbed through the gastrointestinal tract and strongly bind to hydroxyapatite molecules in areas of active bone resorption[119]. The use of BPs is of interest due to potential integrity and welfare issue associated with this class of drug in the horse[120,121].

There are two classes of BPs: non-nitrogenous and nitrogen containing, with each class having distinct modes of action[118,122]. Amino-based BPs such as zoledronic acid (ZA) and alendronate are known to be more potent inhibiting the farnesyl pyrophosphate synthase (FPPS) mechanism which is integral in the mevalonate/cholesterol biosynthetic pathway for bone resorption purposes[122]. Nitrogenous bisphosphonates such as ZA have the strongest affinity for hydroxyapatite with preferential localisation at sites of high bone turnover, are extremely potent and are commonly used in humans for the management of skeletal complications such as malignant and benign bone diseases (e.g., osteoporosis, Paget's disease, and metastases of solid tumors[123,124]. The potency of the nitrogenous bisphosphonate is likely due to the carbon atom that contains the nitrogen-containing side chain[123]. The use of nitrogenous bisphosphonates prevents bone resorption thereby increasing the bone mass and decrease in skeletal fractures. Unfortunately, one of the major side effects in the human use of nitrogenous bisphosphonates is the association of osteonecrosis in the jaw which can occur to 10% of patients who are prescribed ZA[125]. In the equine, ZA has been used for the treatment orthopaedic conditions including bone edema, pain and bone fragility disorders[123].

Comparatively non-amino based BPs such as tiludronic acid (TA), clodronate and etidronate inhibit the function of osteoclasts potentially interfering with adenosine triphosphate (ATP) in any one system and decreasing the amount of bone resorption in the horse[118,126].

Compared to ZA, non-nitrogenous BPs do not have the hydroxyl group on the carbon joining to the two phosphorus groups causing the lack of potency and bone specificity[123]. Currently in the USA, only two non-amino based BPs (tiludronate and clodronate) are approved for the treatment of Navicular syndrome, a major cause of forelimb lameness in horses[122]. A study conducted by Denoix *et al.* concluded that horses treated with a higher dose of TA showed optimal improvement of lameness and returned to normal levels of activity 2-6 months post-treatment using a 1 mg/kg dose[127]. Comparatively, with a lower dose, this failed to significantly improve the condition of the horses examined[127]. According to the requirements set out by the International Agreement on Breeding, Racing and Wagering (IABRW) by the International Federation of Horseracing Authorities (IFHA), approved BPs are not to be administered to racehorses that are under the age of 3 years and 6 months to not interfere with the natural growth of the skeleton for younger horses[121,122]. At present, there are no nitrogen-containing BPs that are approved for use in racehorses and under the rules of racing therefore, the presence of these in any doping control sample constitutes a prohibited substance finding[91,122].

The use of BPs in racehorses has the potential to increase bone mass[128]. However, in horses, bone mass is extremely difficult to measure reliably as horses cannot be told to stand or sit, unlike humans. There is preliminary evidence that there are bone markers which can be monitored to measure the effects of BPs and bone turnover in horses[128]. Such bone markers for the monitoring of BPs but not exclusively include the terminal cross-linking telopeptide of type I collagen (CTX-1) marker for bone resorption[129]. There is also increasing evidence that BPs may also have analgesic effects that act at central and peripheral levels posing an integrity issue for the racing industry[119].

Currently the only method of detecting CTX-1 is the use of enzyme-linked immunosorbent assay (ELISA) with ELISA kits available for purchase commercially. These assays were developed for three forms of collagen type-1 telopeptides including CTX-1[130]. In human studies, the assay is convenient to use and is apparently sensitive to bone resorption changes during diseases however it is not as sensitive for osteoporosis[131]. The monitoring of CTX-1 in animals has not be assessed in full details but in humans, assays can be stored at below -20°C for at least 6 months in addition, samples must not undergo multiple free thaw cycles[130].

Whilst a racing laboratory could potentially use this assay, there are many limitations one faces with such assay. One such limitation is the cost of the CTX-1 assays, the assays are expensive with horse based CTX-1 ELISA kits costing an upward of $500 AUD each. Many racing laboratories could not continuously purchase such expensive kits. Secondly, the need to obtain an ELISA instrument if one does not utilise one already which would be expensive to obtain and require specialist training and analysts for usage. Therefore, the need for an alternate form of detection using endogenous biomarkers would be ideal to detect for the administration of BPs.

Popot *et al.* explored the pharmacokinetics of TA in horses using a field population study in 2017 comprising 39 horses with clinical signs of any specific bone condition for 40 days. The authors concluded that a horse's activity did have a significant effect on the half-life of TA[126]. For horses that were not exercising, the half-life was 289 h whilst horses that were exercising had a half-life of 370 h[126]. With the data collected from this study, the plasma TA concentration in 96% of the horses was evidently lower than 10 ng/mL after 25 days[126]. This study also predicted that on average, for another 25 days, the concentration of TA should be lower than 0.2 ng/mL. Therefore, providing a distinct time of withdrawal is difficult[126]. Additionally, veterinarians have to be aware of the different half-life for horses that are administered TA as to whether they are exercised regularly or not[126]. This work conducted by Popot provided an understanding of TA pharmacologically in the horse to assist in racing authorities and veterinarians in establishing a withdrawal time (and possibly individual detection times) for the use of TA[126].

There are numerous studies conducted that focus on the elimination of the BPs in the horse, however an overarching issue is the difficulty in the extraction of BPs. A study conducted by Popot *et al.* in 2014 explored the extraction and analysis method for non-amino BPs for the detection of TA (Tiludronate or *Tildren*) in equine plasma[89]. As BPs are extremely hydrophilic and have a strong affinity to bone, matrix effects are a major factor in the difficulty to detect BPs using generalised extraction methods[89]. The authors also discovered that detection in urine was inconsistent therefore, the study was only conducted in plasma.

A study conducted by Wong *et al.* in 2015 explored the extraction and analysis method for five separate BPs in equine urine and plasma. As BPs are extremely hydrophilic and ionise extensively in aqueous solutions, simple liquid-liquid extractions are ineffective in addition to matrix effects[91]. BPs are also known to be poorly retained on reverse phase columns, therefore the majority of analytical techniques commonly used in racing laboratories are not appropriate unless derivatisation is performed[91]. This paper explored the use of various derivatisation techniques including diazomethane, trimethylsilyl diazomethane (TMSD) and trimethyl orthoacetate (TMOA). Out of these three derivatisation techniques, TMOA did not produce satisfactory sensitivity for one of the targeted BPs suggesting that whilst it isn't optimum for a range of BPs, it a specific BP confirmation is required, it is possible to use TMOA. The authors concluded from the method that TMSD is optimal for a large range of BPs given that there is a commercially available ether solution, and it was a highly effective methylating agent for water soluble BPs, however, the use of TMSD is highly toxic[91].

Wong *et al.* also noted that during method development, that matrix effects would contribute to reduced robustness and recovery of the BPs. Using a mixed-mode weak anion exchange cartridge, moderate recoveries were obtained for TA in urine and aledronic acid in plasma whilst the other BPs (Clodronate, Ibandronic sodium monohydrate and Risedronate) produced low recoveries but were still consistently detected[91]. As the authors were concerned about the low recoveries, a polymeric sorbent was included in the method prior to the weak anion exchange cartridge which saw an improvement[91].

A study conducted by Popot et al. was performed to develop a method for the detection of non-amino BPs. Due to the complex and polar nature of BPs, a derivatisation step using TMOA was necessary for analysis of TA[89]. Using this method, levels of TA showed a decreasing trend from 3 h post-administration (less than 1 µg/mL), to 24 h post-administration (less than 100 ng/mL) to 15 days post-administration (less than 10 ng/mL)[89]. After 30 days post administration, TA was less than the limit of quantification[89]. The study conducted by Wong *et al.* also explored the use of the method developed with a TA administration which saw a maximum concentration of TA at 9.3 µg/mL at 0.1 hours post-administration with detection possible until 7 days post-administration in urine and up to 1 day post-administration in plasma (plasma samples were only collected up to 1 day post-administration)[91].

BPs have the ability to persist in the bone for several years after administration as they have the capability to form hydroxyapatite crystals prior to being fully absorbed by active osteoclasts where their inhibitory action is performed[129]. A study conducted by Riggs *et al.* in 2020 explored the concentration of TA in 24 horses following administration from approximately 1 month to over 3 years prior to date of sample collection. TA was detected in urine and plasma samples from all administered horses, including two which were administered TA more than 3 years prior to this study[121]. In plasma, the average concentration of TA between 37 to 76 days post-administration was 2.6 ng/mL, between 286 to 335 days was 0.40 ng/mL and for more than 2 years an average concentration of 0.21 ng/mL[121]. Comparatively, in equine urine samples, concentration of TA was inconsistent but those that could be quantified displayed concentrations generally lower than equine plasma[121]. From this study, the authors concluded that TA has the potential to be detected over 3 years after the original administration. This could have serious ramifications for the racing industry[121].

The use of ZA in horses, however, has not been explored as in depth as TA given its lack of approval for usage. A study conducted by Nieto *et al.*[123] explored the pharmacokinetics, pharmacodynamics, and the safety of use of ZA in the horse. For each horse, 0.057 mg/mL was administered to each horse for 30 minutes with blood collected at 1, 5, 10, 15, 20, 30, 45, 60 and 90 minutes and 2-, 3-, 4- and 8-hours post-administration. Samples were also collected 1, 4, 7 and 28 days, 9 weeks, 6 months and 1 year after administration. From this administration, the authors observed no adverse effects with the administration with concentrations of ZA decreasing rapidly after administration with 8 hours showing no detectable plasma concentrations of ZA[123]. The authors hypothesised that due to the hydroxyl groups on the carbon atom, this is causing the poor metabolism and high affinity for bone structures[123]. Nonetheless, the authors concluded that ZA is the strongest BPs currently available with the benefits still requiring further studies.

5.1.1 Aims

Currently in the equine anti-doping industry, the elimination of TA has been extensively explored with numerous studies looking at the elimination in addition to the methods of extraction for TA. Comparatively, the elimination of ZA has not been explored in as much

details as TA due to its prohibited status. Therefore, the aims for the BP were to firstly, determine the detection period for TA and ZA using a novel method. Secondly, determine if there are any lipid and corticosteroid biomarkers that could either complement or indirectly extend the time of detection of a particular BP. Additionally, whether the use of individual reference limits for the biomarkers could allow for indirect detection of BPs. The last aim was to determine if there are any biomarkers which display differential presence in either one of the BPs in order to distinguish between a nitrogenous and a non-nitrogenous BP.

5.2 Methods

5.2.1 Chemicals and reagents

LC grade acetonitrile (ACN), hydrochloric acid (HCl), methanol (MeOH), formic acid (FA), triethylamine (TEA) and TMOA were purchased from Merck (Castle Hill, NSW, Australia). LC grade glacial acetic acid was purchased from ThermoFisher (Waltham, Massachusetts, USA) and water (H_2O) used was ultrapure grade (18.2 MΩ cm) obtained from a ThermoFisher Barnstead Smart2Pure system (Langenselbold, Hungary).

Two sources of TA were obtained for either calibration or quality control. For calibrators, TA was manufactured and purchased from Merck (Castle Hill, NSW, Australia). For quality control spikes and the corresponding internal standard, TA and TA-D_5 was manufactured from Toronto Research Chemicals (Toronto, Ontario, Canada) and purchased from PM Separations (Capalaba, QLD, Australia). ZA for calibration and quality control spikes was manufactured and purchased from Merck (Castle Hill, NSW, Australia). The corresponding internal standard of ZA-$^{13}C_2$-^{15}N, was manufactured by Toronto Research Chemicals (Toronto, Ontario, Canada) and purchased from PM Separations (Capalaba, QLD, Australia).

5.2.2 Administration Study – Zoledronic Acid

An 8-horse study comprising of 4 males and 4 geldings of ZA (Permit number: 81385. Randlab, Chipping Norton, NSW, Australia) was completed through IV administration. A dose of 0.057 mg/kg of ZA was administered by a 30-minute IV infusion using 1 L of saline through the jugular vein using a catheter in the neck of the horse. Blood samples were taken via the

opposite jugular vein to avoid cross contamination. Blood samples were collected from 24 hours prior to administration, 0 (time of administration), 1, 5, 10, 15, 20, 30, 45, 60, 90 minutes, 2, 3, 4, 6, 8, 12 hours, 1, 2, 3, 4, 5, 6, 7-, 14-, 21- and 28-days post infusion. Blood samples were taken after the entire 30-minute infusion had been given to each horse. Sampling that was collected once a day was completed at 8 am for the respective day. Blood samples were immediately centrifuged following collection at 1,500 x g for 10 minutes to obtain the plasma layer. Plasma samples were then stored at -20°C until extraction and analysis. Samples from this administration underwent one freeze thaw cycle. Animal ethics approval (A20062) was obtained for this study from Charles Sturt University Animal Care and Ethics Committee.

5.2.3 Administration Study – Tiludronic Acid

An 8-horse study comprising of 4 males and 4 geldings with *Tildren®* (Tildren, Ceva Animal Health. Glenorie, NSW, Australia) was completed. A 1.0 mg/kg dose of *Tildren®* was administered by a 30-minute IV infusion using 1 L of saline through the jugular vein using a catheter in the neck of the horse. Blood samples taken via the opposite jugular vein to avoid cross-contamination. Blood samples were collected 6, 5, 4, 3, 2, and one day prior to administration of TA to establish baseline values as these horses were previously administered ZA 12 months earlier. The baseline values were to determine whether the ZA was still influencing the bone markers. Blood samples were then collected at time 0 (time of administration), 1, 5, 10, 15, 20, 30, 45, 60, 90 minutes, 2, 3, 4, 6, 8, 12 hours, 1, 2, 3, 4, 5, 6, 7-, 14-, 21- and 28-days post-infusion. Blood samples were taken after the entire 30-minute infusion had been given to each horse. Sampling that was collected once a day was completed at 8 am for the respective day. Blood samples were then immediately centrifuged at 1,500 x *g* for 10 minutes to obtain the plasma. Plasma samples were then stored at -20°C until extraction and analysis. Samples from this administration underwent one freeze thaw cycle. Animal ethics approval (A21362) was obtained for this study from Charles Sturt University Animal Care and Ethics Committee.

5.2.4 Surrogate Matrix

A surrogate matrix was utilised due to the endogenous nature of the target lipid and corticosteroid compounds. This method is outlined in a previous chapter 2 – Lipids.

5.2.5 Lipid Extraction

Outlined in Chapter 2 - Lipids.

5.2.6 Bisphosphonate Extraction

The method utilised was adapted from Popot *et al.* and Wong *et al.* and is the National Association of Testing Authorities (NATA) accredited qualitative method at the Australian Racing Forensic Laboratory (ARFL) in accordance with the ISO/IEC 17025 standard[89,91]. An aliquot of 1 mL of equine plasma was obtained with relevant Internal standard (IS) added at a concentration of 20 ng/mL for TA-D_5 and 50 ng/mL for ZA-$^{13}C_2$-^{15}N. Samples were pH adjusted to 4 using 2 mL of buffer containing diluted HCl in H_2O (pH of 2) then centrifuged at 3000 rpm for 10 minutes.

Solid phase extraction (SPE) was completed on the Biotage Extrahera Classic (Uppsala, Sweden) using two separate cartridges. The first SPE was performed using a Waters Oasis® HLB (60 mg 3 mL) cartridge (Milford, Massachusetts, USA) for TA or an Agilent Bond Elut Polypropylene (PPL, 100 mg, 3 mL) cartridge (Santa Clara, California, USA) for ZA. The cartridge was conditioned using MeOH (2 mL) and H_2O (2 mL) prior to samples being loaded and collection of the flow-through for the second SPE. The second SPE for both ZA and TA used a Waters Oasis® WAX (60 mg, 3 mL) cartridge (Milford, Massachusetts, USA). The cartridge was conditioned with MeOH (2 mL) and acidified water (pH adjusted to 4 with FA; 2 mL). Sample were loaded then washed with acidified water (pH adjusted to 4 with FA; 2 mL) followed with MeOH (2 mL). Cartridges were dried for 2 minutes before target compounds were eluted with 15% TEA in MeOH (3 mL). The eluent was dried under nitrogen gas at 60°C.

Samples were then derivatised using acetic acid and TMOA with heating at 95°C for 60 minutes before being dried under nitrogen gas at 60°C. Samples were reconstituted in 50:50

MeOH and H_2O (100 µL) and stored at 4°C until liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis.

5.2.7 Instrument Parameters for Lipids

Outlined in Chapter 2.2.6.

5.2.8 Data Analysis Parameters for Lipids

Outlined in Chapter 2.2.8.

5.2.9 Instrument Parameters for Bisphosphonates

Using the NATA accredited qualitative method for BP analyses, LC-MS/MS analysis was undertaken with a LC30 liquid chromatograph system coupled to an 8050-mass spectrometer from Shimadzu Scientific Instruments (Kyoto, Japan). Separation was performed using a Waters XBridge C18 column (2.1 mm x 150 mm, 3.5 µm) (Milford, Massachusetts, USA) using a gradient elution. Aqueous mobile phase A consisted of 0.1% FA in H_2O whilst organic mobile phase B was 0.1% FA in ACN with a run time of 14 minutes. The gradient was: 0-1 minute B (2%), 1-8 minutes B (98%), 8-12 minutes B (2%) and then kept constant until 14 minutes. The flow rate was constant at 0.2 mL/min with an injection volume of 5 µL and the column oven was set to 35 °C.

Shimadzu LabSolutions software (version: 5.93) was used for data acquisition using a National Association of Testing Authorities (NATA) accredited method at the ARFL. Data was acquired using multiple reaction monitoring (MRM) in ESI positive mode. The MS conditions are stated in appendices table A17.

5.2.10 Method Validation of Zoledronic Acid and Tiludronic Acid

The established LC-MS/MS method for the quantification of ZA and was validated for 1 mL of equine plasma. The parameters assessed were discussed in Chapter 2.2.7 Method Validation Preparation.

5.2.10.1 Linearity

Linearity was assessed as described in chapter 2.2.7.1 with batches analysed from R^2 value using data obtained from 3rd March 2023 to 18th of August 2023 with 4 batches used. Concentrations of 0, 0.5, 1, 2, 5, 10, 20, 50, 100, 200, 500, 1000 ng/mL were used for both TA and ZA. A working solution of 2000 ng/mL was prepared from a stock solution of 954.5 µg/mL for TA and 907.83 µg/mL for ZA. From the working solution, a further dilution was completed to 200 ng/mL and 20 ng/mL to spike into equine plasma. The spike amounts for each calibration were determined using equation 2.1 from chapter 2.2.7.1: Linearity. The amount for each concentration is provided in appendices Table A18. Regression analysis was also completed in accordance with chapter 2.2.7.1.

5.2.10.2 Sensitivity

The method sensitivity was assessed by estimating the LOD and LOQ as set out in chapter 2.2.7.2 for both ZA and TA. Using the signal-to-noise (*S/N)*, the comparison could then be made for a *S/N* of 3 for the LOD and a *S/N* of 10 for the LOQ^{100}. Concentrations of 0.5, 0.4, 0.3, 0.2, 0.1 and 0.05 ng/mL were chosen for TA whilst 20, 10 and 5 ng/mL were chosen for ZA. Table A19 and Table A20 in the appendices provides the total amounts required for each spiking solution for ZA and TA respectively.

5.2.10.3 Accuracy

Accuracy was assessed in a similar manner to those as set out in chapter 2.2.7.3 and using Equation 2.3. Concentrations were estimated using plasma spikes at 50 ng/mL for ZA and 10 ng/mL for TA.

5.2.10.4 Precision

Precision was estimated in a similar manner to chapter 2.2.7.4 and using Equation 2.4. Concentrations used for precision are the same as accuracy (50 ng/mL for ZA and 10 ng/mL for TA.

5.2.10.5 Recovery

Recovery was assessed in a similar manner to chapter 2.2.7.5 using Equation 2.5. Concentrations used for recovery was the same as accuracy and precision (50 ng/mL for ZA and 10 ng/mL for TA).

5.2.10.6 Matrix Effects

Matrix effects (ME) were assessed using the conditions as described in chapter 2.2.7.6 and using Equation 2.6. Concentrations used for ME was the same as accuracy, precision, and recovery (50 ng/mL for ZA and 10 ng/mL for TA).

5.2.10.7 Dilution

Dilution was assessed using 7 replicates representing either a 1 in 10 dilution for TA or a 1 in 2 dilution for ZA. The chosen concentration for the spike was 200 ng/mL for TA and 100 ng/mL for ZA. These concentrations were chosen as the sample volume would then fall within the chosen calibration ranges. The concentration was firstly determined by the instrument calibration and then multiplied by relevant dilution factor to gain the actual concentration of the sample. Concentrations were then calculated to obtain the % RE.

5.2.10.8 Stability

Stability was assessed over a 4-week period with samples analysed at 2-weeks, 3-weeks 3-month for ZA whilst for TA, samples were analysed at 1 month, 2 months and 3 months. Concentrations were the same concentrations as previous method validation requirements and were stored at either 4°C or -20 °C. At the allocated time point, the samples were thawed to room temperature and prepared using the method as outlined in chapter 5.2.6. Concentrations were calculated utilising the LabSolutions Insight software. The average concentration for each temperature was taken over their associated time period using the average function in *Excel*.

5.2.11 Data Analysis Parameters for Bisphosphonates

All data was processed using Shimadzu Insight software (version 3.2) with further data processing completed on Excel (version: 16.71). This method includes each compound, the precursor and product ion for MRM transition and the internal standard used for compound quantification. The concentrations from the instrument were calculated using linear regression with the calibration curve being the area ratio of the target compound to the internal standard response for the respective concentration. Table A21 in appendices shows the conditions LabSolutions Insight had for the quantification of the compounds.

5.2.12 Equine Biological Passport – Individual Reference Limits

Outlined in Chapter 2.2.9.

5.3 Results and discussion of method validation of Zoledronic Acid and Tiludronic Acid

5.3.1 Optimised Liquid Chromatography and Mass Spectrometry Conditions

LC optimisation was performed to verify chromatographic separation of the target compounds. This was completed with the selection of MRM transitions for the target compounds, related to the structure of each compound. The retention time for each compound was checked using the highest calibrator for each sequence with the expected retention time to fall within a ± 0.1 minutes of the corresponding reference material. Relative retention time was calculated as per the equation in chapter 2.3.1 using Equation 2.13. The relative retention was also verified by comparing the reference material retention time to the respective internal standard. According to Association of Racing Chemists (AORC) criteria, the acceptable range for the relative retention is 2% for liquid chromatography (LC) conditions[101]. The optimised conditions for the LC for the bisphosphonates and its relative internal standard as provided in Table 5.1 whilst the optimised MS conditions for each compound is provided in Table 5.2.

Table 5-1: Optimised LC retention times for bisphosphonates

Compound	**Retention Time (minutes)**	**Relative Retention**
Zoledronic Acid	4.67	1.00
Zoledronic Acid - $^{13}C_2$-^{15}N	4.67	1.00
Tiludronic Acid	7.20	1.00
Tiludronic Acid – D_5	7.19	1.00

Table 5-2: Optimised MS Conditions for bisphosphonates (* indicates product ion used to quantify).

Compound	**ESI (+/-)**	**Precursor ion (*m/z*)**	**Product Ion (*m/z*)**	**Collision energy (CE)**
Zoledronic Acid	Positive	328.80	202.90*	-17*
			135.00	-13
			171.10	-17
Zoledronic Acid - $^{13}C_2$-^{15}N	Positive	333.10	206.90*	-21*
			136.95	-30
Tiludronic Acid	Positive	375.00	342.85	-17
			157.00	-21
			216.95*	-21*
			154.80	-44
Tiludronic Acid – D_5	Positive	378.90	161.00*	-22*
			86.95	-22

5.3.2 Linearity

TA required two separate calibration curves to accurately quantify either the low or high end of the calibration curve. Figure A10, A11 and A12 in the appendices shows the calibration curves for ZA (A10) and TA (A11 and A12) with Table 5.3 providing the R^2 values for TA and ZA.

Table 5-3: R^2 values for Tiludronic Acid and Zoledronic Acid in equine plasma.

Compound	R^2 value (averaged)
Zoledronic Acid	0.991
Tiludronic Acid (0-200 ng/mL)	0.998
Tiludronic Acid (0-1000 ng/mL)	0.981

Further assessment of linearity was completed using the y-residuals with the same 4 batches used. From the y-residual plots, this showed no bias in the data from the random placements of the residual data points for each concentration.

5.3.3 Sensitivity

The LOD for TA was estimated to be 0.5 ng/mL and ZA was 10 ng/mL. For LOQ, TA was estimated to be 1.0 ng/mL and ZA was 20 ng/mL.

5.3.4 Accuracy, Precision, Recovery and Matrix Effects

Table 5.4 provides the results for accuracy and precision with determined concentrations needing to be equal to or less than 25% to be considered precise or accurate. Both TA and ZA were within the acceptable accuracy and precision conditions. Recovery for this extraction was poor for TA (13.2%) and ZA (2.70%) which is also a contributing factor to the high LOD and LOQ for ZA. A major reason is likely the use of an inefficient cartridge as the second SPE. The current second cartridge used was the Waters Oasis® WAX (60 mg, 3 mL) cartridge in accordance with the method published by Wong *et al*[91]. According to unpublished results by Klingberg *et al.* (personal communication) an improved cartridge for bisphosphonate

extraction is the Affinisep AttractSPE®WAX (Normandy, France). The exact reason for such low recovery remains unknown, however, it may be likely due to the low pH which the sample are isolated on the cartridge. A higher pH (e.g. 4 to 6) could be investigated for improved recoveries. Both compounds showed only slight ion suppression.

Table 5-4: Accuracy, precision, recovery and matrix effects for TA and ZA

	Concentration (ng/mL)	Accuracy (% RE)	Precision (% RSD)	Recovery (%)	Matrix Effects (%)
Zoledronic Acid	50.0	9.80	6.80	2.70	95.0
Tiludronic Acid	10.0	6.38	13.2	13.2	82.3

5.3.5 Dilution

Dilution was assessed for ZA and TA concentrations exceeding the highest calibrator of 1000 ng/mL. Table 5.5 shows the results obtained with both of the dilution factors (1 in 10 for TA and 1 in 2 for ZA). Dilution for ZA was acceptable however the relative error for TA was slightly higher. This could be due to lower concentration used for TA compared to ZA. In addition, the low recovery as seen in chapter 5.3.4 could be a contributing factor to the higher dilution effects

Table 5-5: Dilution assessment for bisphosphonates

Dilution	Average Dilution Concentration (ng/mL)	Actual Concentration (ng/mL)	% RE
1 in 2 (Zoledronic Acid)	46.8	93.6	6.43
1 in 10 (Tiludronic Acid)	15.4	154	23.2

5.3.6 Stability

ZA was completed over a 4-week period with samples being tested at time 0, 2-weeks, 3-weeks, and 4-weeks. The stability of TA was assessed over a 3-month period with samples being tested at time 0, 1-month, 2-months, and 3-months. Samples outside of a 20% margin were considered unstable[99]. Tables 5.6 and 5.7 presents the results for ZA and TA respectively at both temperatures of 4°C or -20 °C whilst Figures 5.1 and 5.2 displays these results. There is evidence of inconsistencies between the chosen testing time points for both ZA and TA. For ZA, between 2 to 3 weeks it increases from 87.2% to 113%. At -20°C for TA, the calculated percentage remaining is 116% at 2 months before reducing to 19% at 2 months. This is likely due to the recovery issues as stated in chapter 5.3.4 where TA saw recovery of only 13.2% and ZA 2.70%. This severely limits the overall interpretation of the stability due to the small recovery which may explain the inconsistent stability results. Nonetheless, overall, TA shows some degree of degradation for periods whilst ZA, however, was deemed stable throughout the 4-week period.

Table 5-6: Stability results for ZA at 50 ng/mL as percentage remaining.

Temperature (°C)	Time 0 (% remaining)	2 weeks (% remaining)	3 weeks (% remaining)	4 weeks (% remaining
4	100	87.2	131	104
-20	100	73.6	114	105

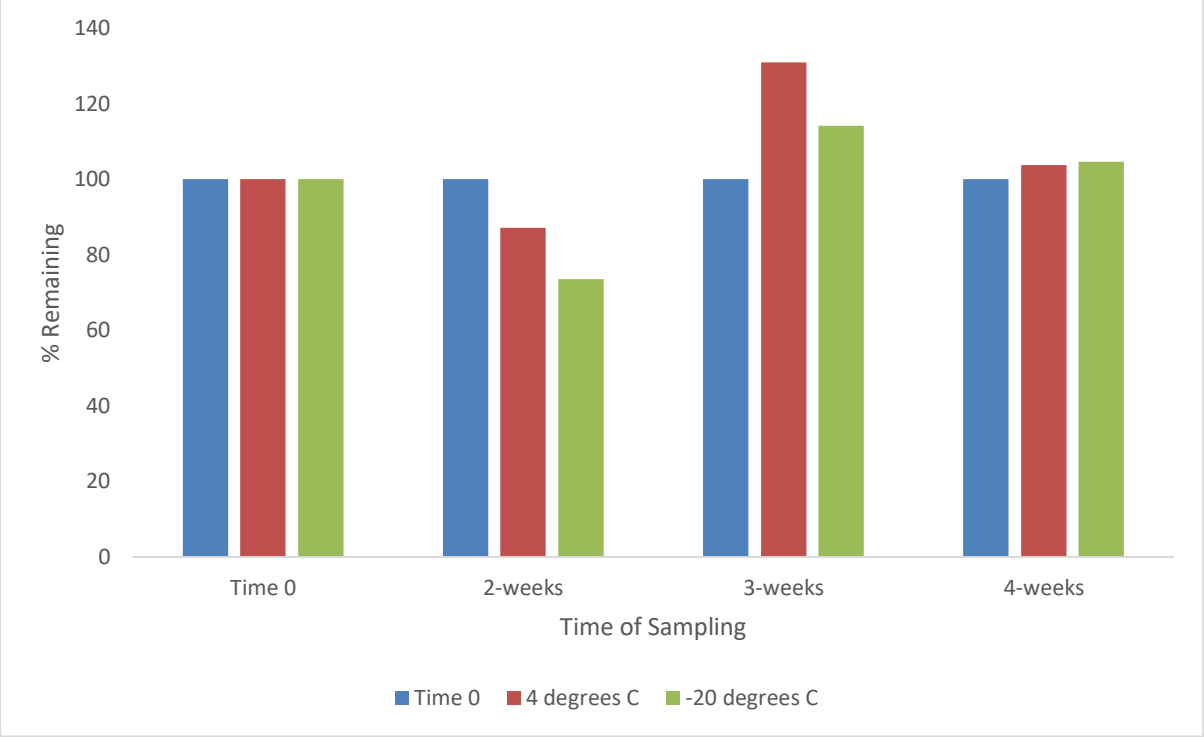

Figure 5.1: Stability for Zoledronic Acid in equine plasma over a 4-week period (completed in duplicate for each time point and temperature).

Table 5-7: Stability results for TA at 10 ng/mL as percentage remaining.

Temperature (°C)	Time 0 (% remaining)	1 month (% remaining)	2 months (% remaining)	3 months (% remaining
4	100	85.2	50.3	14.7
-20	100	116	19.0	57.9

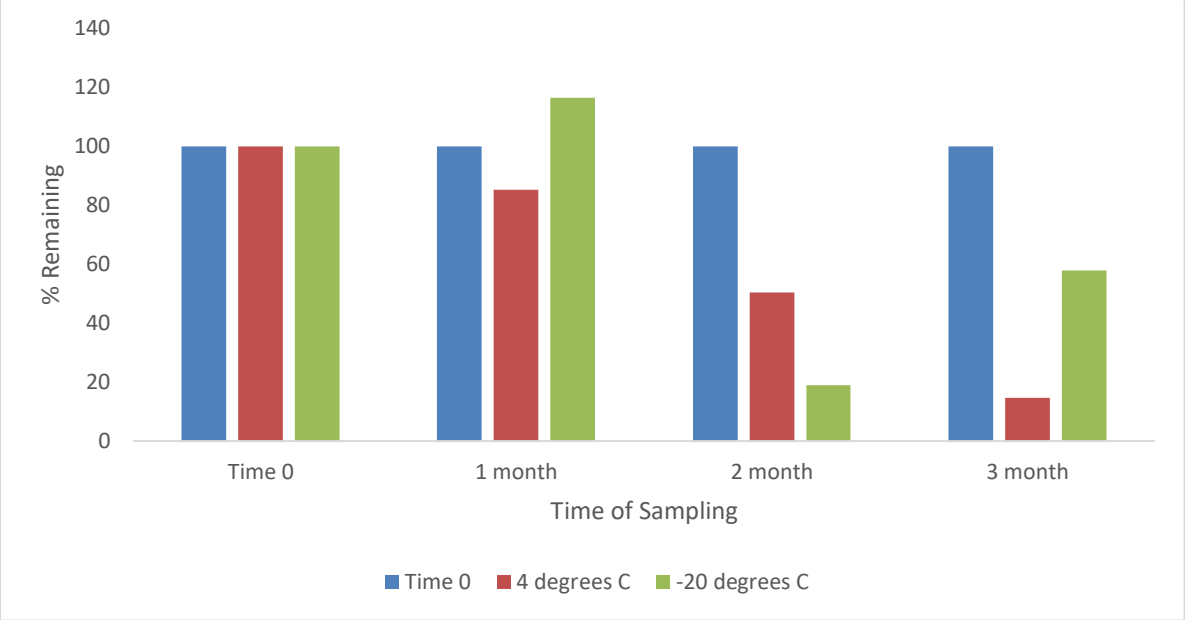

Figure 5.2: Stability for TA in equine plasma over a 3-month period (completed in duplicate for each time point and temperature).

5.4 Results and Discussion from Zoledronic Acid Administration

5.4.1 Targeted Results

5.4.1.1 Zoledronic Acid Quantification

Plasma elimination profiles of ZA in eight horses with four being mares and four geldings are shown in figure 5.3 (for a non-log y scale, refer to appendix figure A13).

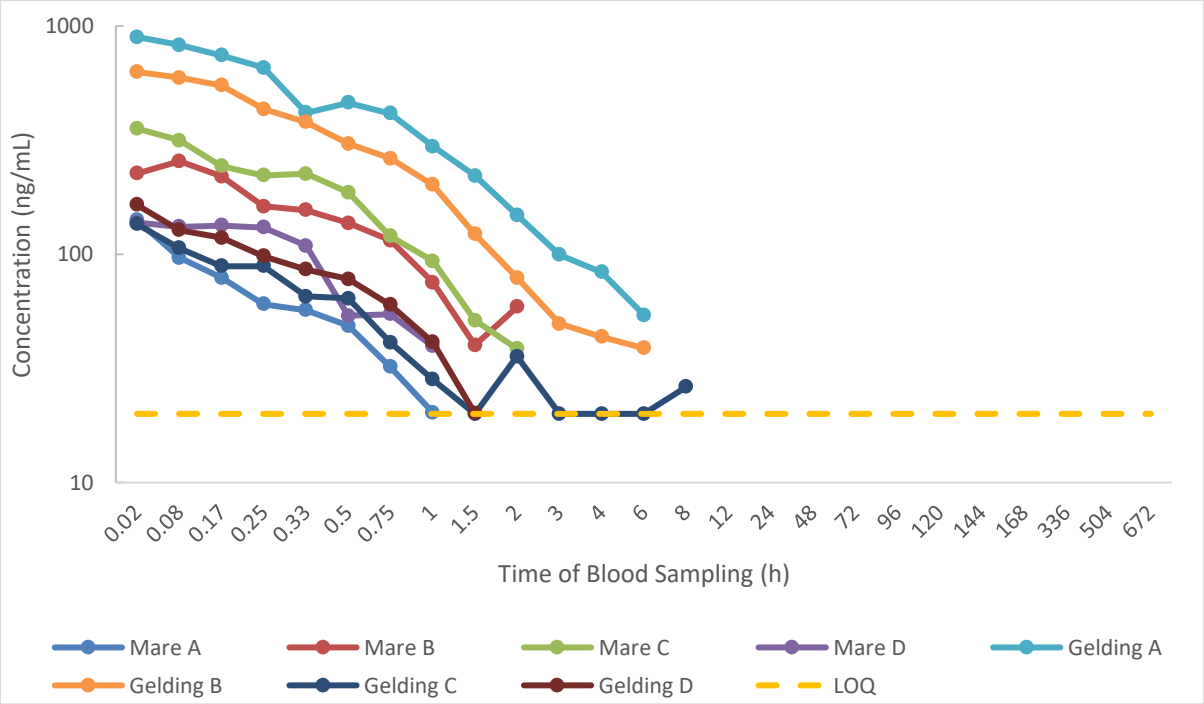

Figure 5.3: Elimination of ZA in 8 administered horses using a log-y scale. The limit of quantification (LOQ) of 20 ng/mL is denoted by a yellow dotted line.

All eight horses exhibited a downward trend of ZA following the maximum concentration usually at 1-minute (0.02 h) post administration however for Mare B, the maximum concentration was at 5 minutes (0.08 h) post-administration. The highest concentration was at 894 ng/mL from Gelding A with the lowest maximum concentration being 135 ng/mL in Gelding C. From this Figure, the last detection point for ZA is 8 hours post-administration which is consistent with the results as published by Nieto *et al.*[123]. There is also evidence of

large inter-individual variation this is likely due to each horse metabolising the drug differently.

This relatively short window of detection highlights the need to search for biomarkers capable of indirectly extending the time of detection for the administration of a nitrogenous bisphosphonate. Under the influence of ZA, the following biomarkers displayed notable change: OEA, HC and HC/C. The biomarkers that remained consistent following ZA administration were AEA, cortisone, 18-hydroxycortisol and 18-HEPE. All other monitored biomarkers were not detected throughout the administration period. A major limitation of this study is the lack of placebo treated (control) horses. Therefore, it cannot be discerned in the biomarker work whether the effect was from ZA and/or from external environmental factors.

5.4.1.2 Lipid and corticosteroid biomarkers

OEA:

Concentrations of OEA were quantifiable throughout the administration period and remained consistent for up to 60 minutes post-administration as seen in figure 5.4 top panel. This was then up-regulated by 337% at 7 days (168 h) post-administration compared to time 0 as seen in figure 5.4 bottom panel. This increase whilst large, had a fold change of 1.8 but unfortunately the p-value in comparison to pre-administration samples was > 0.05 deeming it to be consistent with pre-administration samples. At 14 days (504 h) post-administration however, there was 90% decrease (FC = -0.93, p-value = 0.37) in comparison to time 0 before returning to basal levels. Whilst the samples between 8 hours and 7 days (168 h) did not exhibit the most change in comparison to pre-administration samples, these sample concentrations do exceed the proposed upper population threshold of 5.6 ng/mL as proposed in chapter 2.4.2. Therefore, if these samples were to undergo routine testing, they would be deemed abnormal and would require addition confirmatory testing.

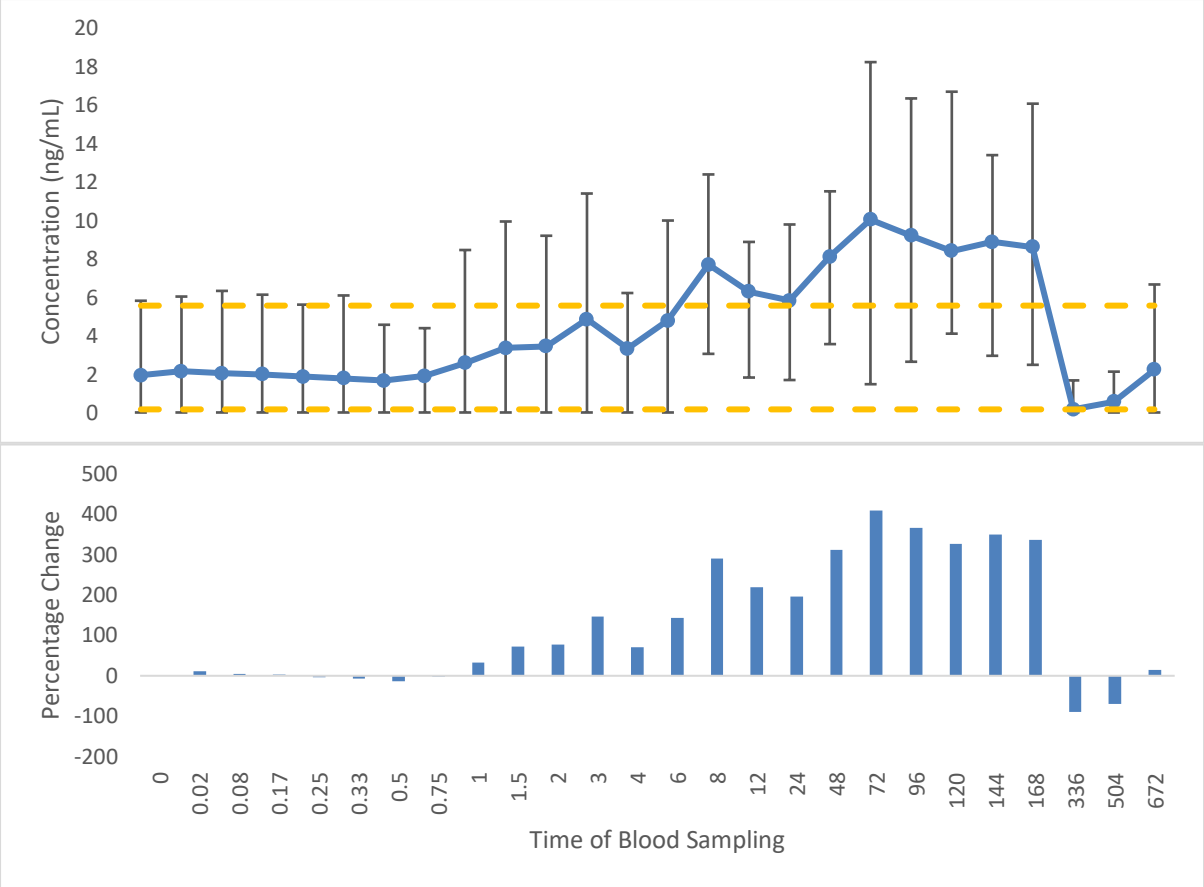

Figure 5.4: Average concentration of plasma OEA following ZA administration. Panel A showing the average concentration, the vertical bars representing the range and the yellow dotted lines representing the proposed PRLs of 5.6 ng/mL and 0.21 ng/mL; panel B showing the average percentage change (n=8).

AEA:

The estimated concentration of AEA remained relatively consistent throughout the administration period as seen in Figure 5.5 top panel never exceeding 0.45 ng/mL. Compared to time 0, the largest change was seen at 4 days (96 h) post-administration with a 237% increase as seen however the FC was calculated to be 1.32 therefore did not show much change not in comparison to pre-administration samples.

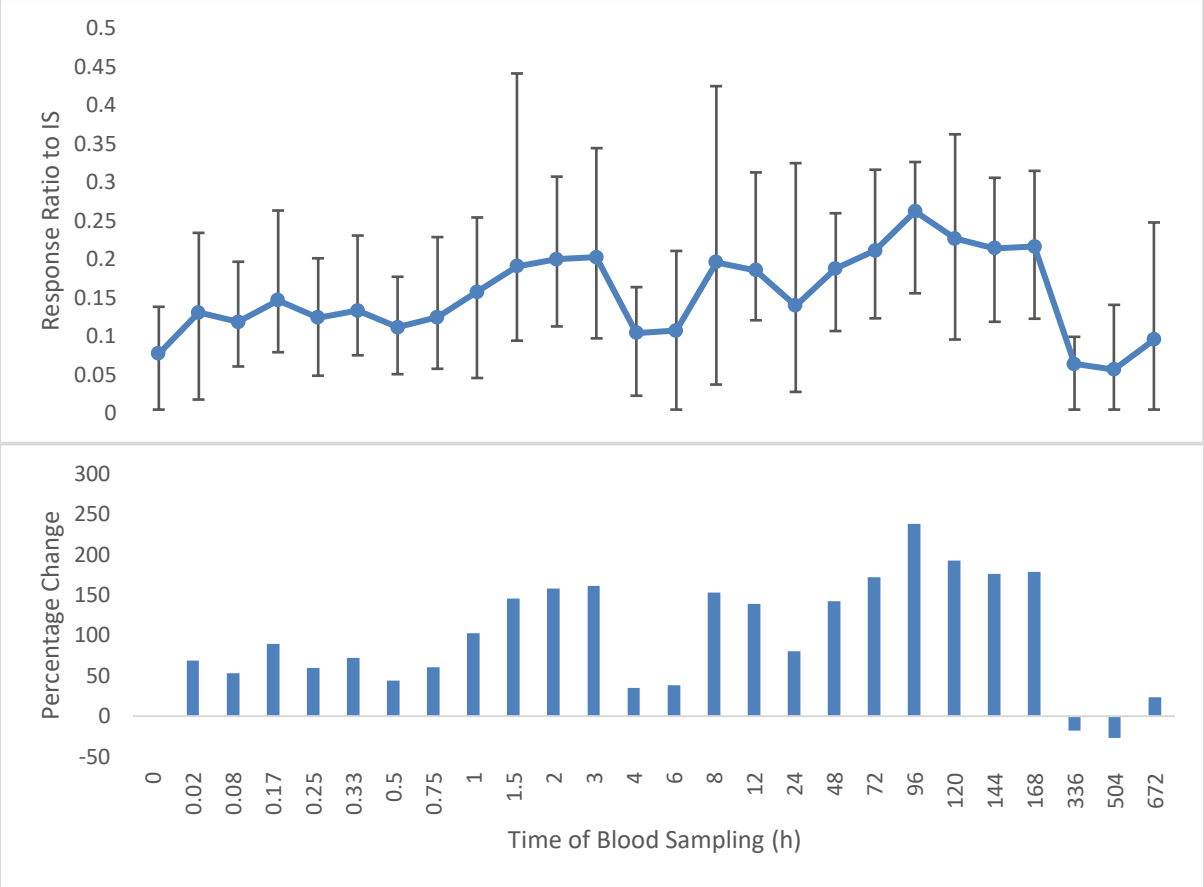

Figure 5.5: Average integrated peak area of plasma AEA following ZA administration. Panel A showing the integrated peak area of AEA with vertical bars representing the range; panel B showing the average percentage change (n=8).

Hydrocortisone:

During ZA administration, the estimated concentrations of hydrocortisone were relatively consistent until 12 hours post-administration where a 48% decrease is seen in comparison to time 0 as seen in Figure 5.6. The FC was calculated to be -0.49 therefore, was consistent with pre-administration samples. Estimated concentrations then returned to basal levels and were consistent until 21-28 (504 – 672 h) days post administration with an increase of 103-112% respectively as seen in Figure 5.6 bottom panel. Unfortunately, the FC was calculated to be 1.00 and 1.01 respectively therefore these two time points are also consistent with pre-administration samples. There is currently no literature that details the possibility of why this may occur however, one hypothesis is with the potency of ZA being 10,000 times more potent than TA[132], the analgesic effect is seen at a much later time point in comparison.

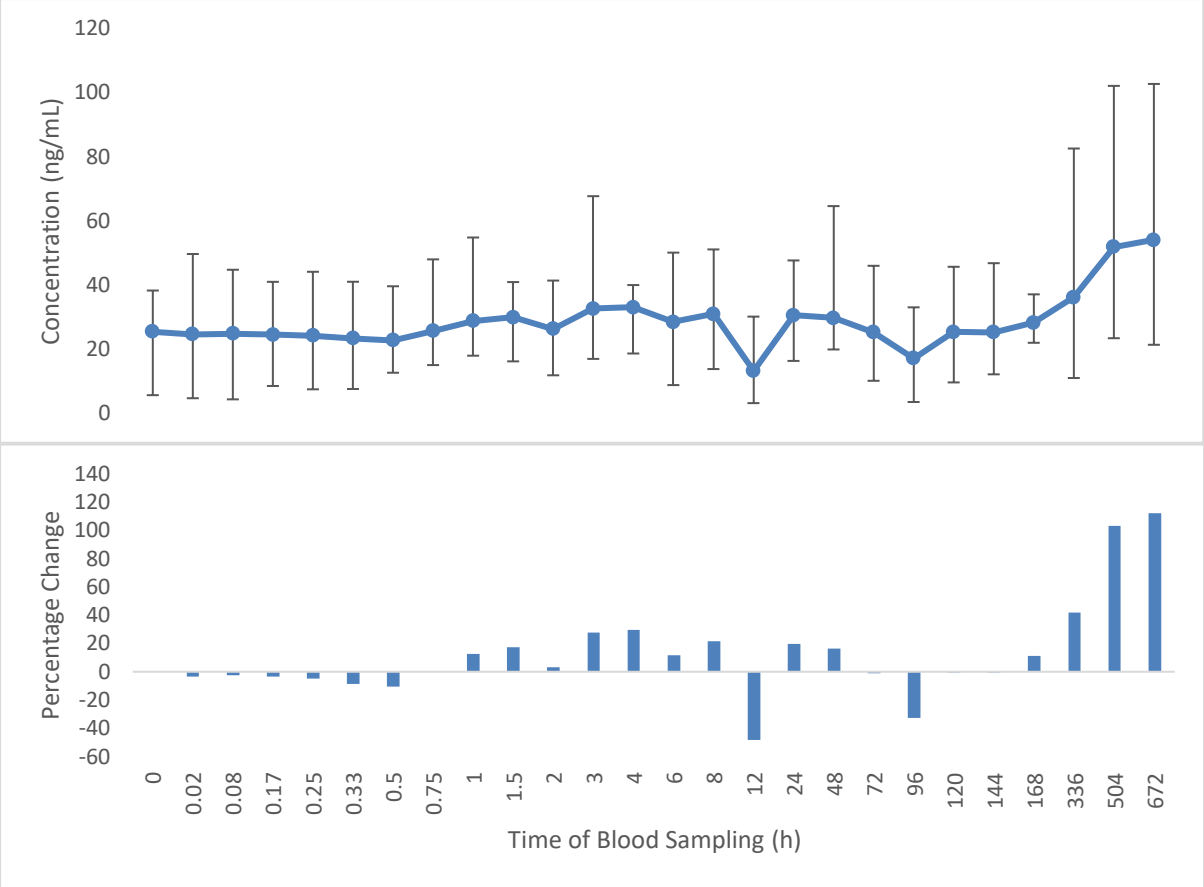

Figure 5.6: Estimated average concentration of plasma hydrocortisone following ZA administration. Panel A showing the estimated average concentration and the vertical bars representing the range; panel B showing the average percentage change (n=8).

Hydrocortisone/Cortisone Ratio (HC/C):

Cortisone was monitored which generated very consistent concentrations never exceeding 2.5 ng/mL allowing cortisone to be used as an ERC. Sampling for horses that occurred once a day was completed at approximately 8 am each day, therefore diurnal variation was not accounted for in this study. The HC/C ratio showed consistency throughout the administration but there is an erratic change with the ratio between 2-8 hours post-administration as seen in Figure 5.7. At 21 days (504 h) post-administration, there was a distinct increase. Looking at the percentage change, the erratic elimination pattern saw a percentage change from 38% at 2 h to 27% increase 3 h post-administration compared to time 0. However, the FC at these two time points was -0.24 and 0.58 respectively deeming these two time-points consistent with pre-administration samples. At 21 days (504 h) post-administration, the HC/C value of

64 also exceeds the proposed upper ratio threshold of 58[93] highlighting this sample would be flagged as abnormal from routine screening. There is a 47% increase in comparison to time 0 highlighting the possibility of having HC/C as a biomarker that can distinguish TA and ZA administrations.

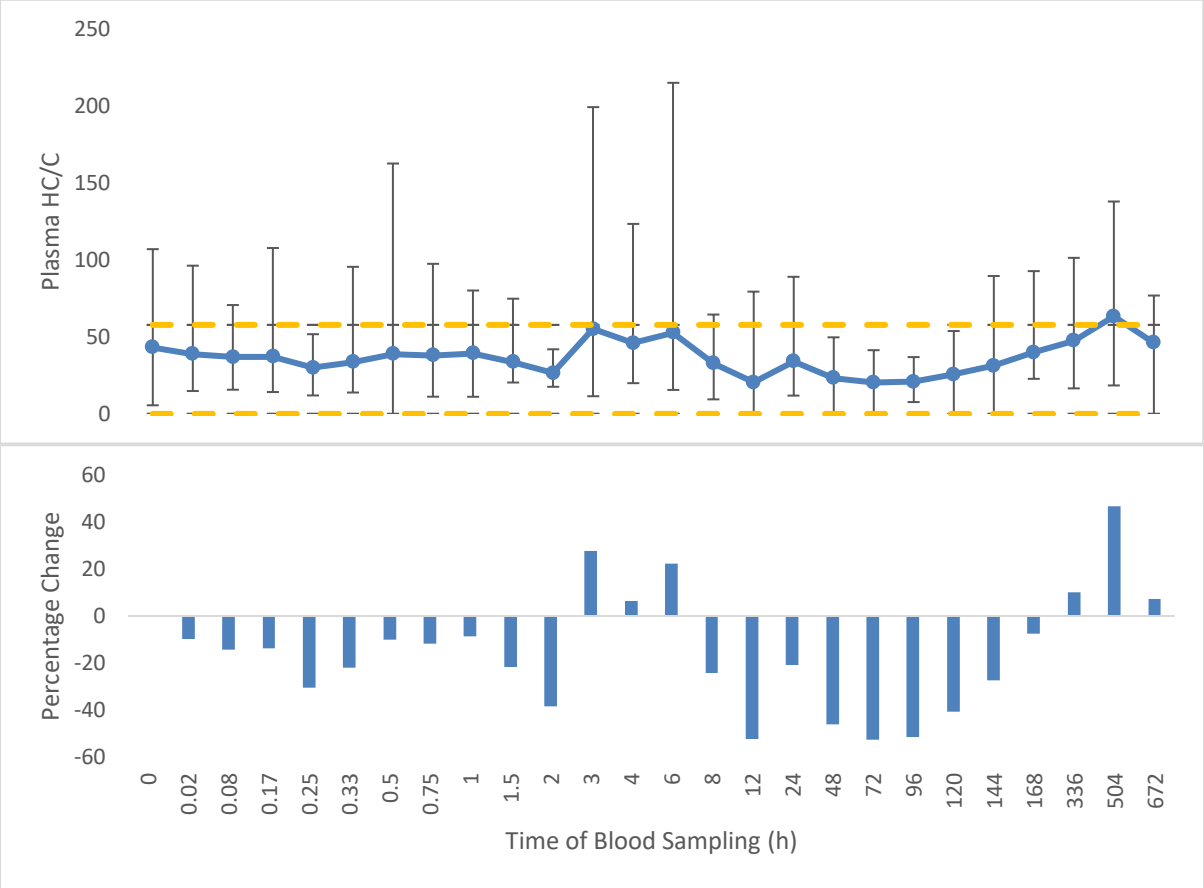

Figure 5.7: Average plasma HC/C ratio following ZA administration. Panel A showing the HC/C ratio and the vertical bars representing the range; panel B showing the average percentage change (n=8).

18-hydroxycortisol:

The biomarker 18-hydroxycortisol during a ZA administration, whilst not quantifiable, it was detectable therefore the area was plotted. As seen in Figure 5.8 top panel, there is no distinct pattern with 18-hydroxycortisol throughout the administration period however, there is increasing levels at 3 hours and 7 days (168 h) post-administration and decreasing levels at 12 h and 4 days (96 h) post-administrations compared to time 0 with majority of the other time points being within 20% compared to time 0 (excluding 5 minutes post-administration

with a percentage change of 37% and 15 minutes post-administration being 35% decreased) as seen in Figure 5.8 bottom panel. However, all these time points had FC < 1 for increasing levels or > 0.67 for decreasing levels therefore, deeming these timepoints consistent with pre-administration samples.

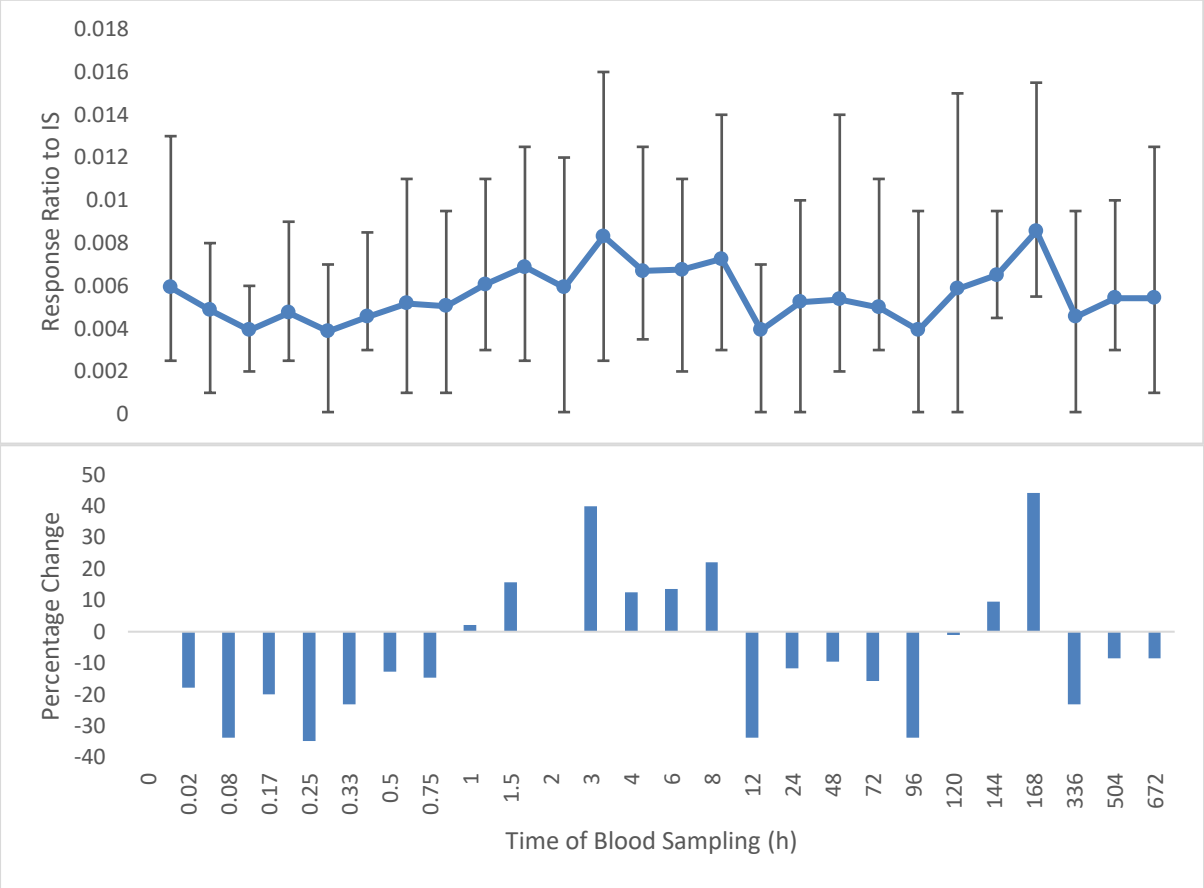

Figure 5.8: Average peak area response for plasma 18-hydroxycortisol profile following ZA administration. Panel A showing the average peak area and the vertical bars representing the range; panel B showing the average percentage change (n=8).

11-deoxycortisol:

11-deoxycortisol showed some potential to being a useful biomarker for the presence of ZA. The one disadvantage is the need for the integrated peak area to be monitored as the concentration could not be quantified. This marker starts off being very consistent until 14 days (336 h) post-administration where a large increase compared to time 0 as seen in Figure 5.9 top panel. Looking at the percentage change in comparison to time 0 (as seen in Figure 5.9 bottom panel), at the 14-day (336 h) time point, this was a 131% increase before dropping

down to a 93 and 81% increase at 21-28 days (504 – 672 h) post-administration. This increase would potentially make 11-deoxycortisol a good marker to monitor for the presence of ZA administration given the lack of the increasing levels at the later time points for a TA administration. However, the FC for all time points were not within the criteria to be deemed as different compared to pre-administration samples. Being the metabolic intermediate to hydrocortisone in the steroidogenesis pathway, having an increased area of 11-deoxycortisol is consistent with also having an elevated concentration of hydrocortisone as previously seen.

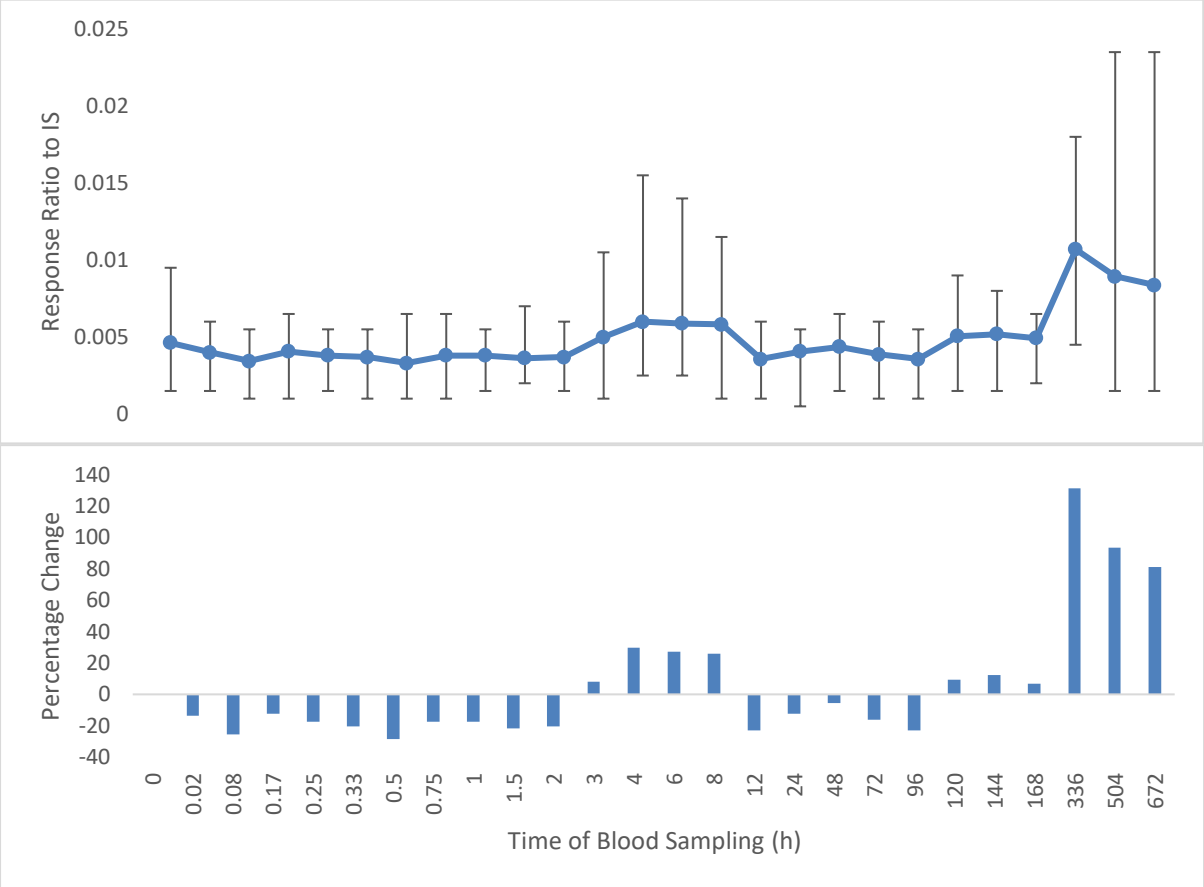

Figure 5.9: Average peak area of plasma 11-deoxycortisol following ZA administration. Panel A showing the mean integrated peak area and the vertical bars representing the range; panel B showing the mean percentage change (n=8).

18-HEPE:

The presence of 18-HEPE was very consistent throughout the administration period. As seen in Figure 5.10 top panel, the concentrations of 18-HEPE never exceeded more than 5 ng/mL. In reference to the percentage change as seen in Figure 5.10 bottom panel, the later time

points remained relatively consistent not exceeding the 20% change (excluding 2-, 3- and 4-hours post-administration that did not exceed 30%) which could potentially see the use of 18-HEPE as an ERC again for the administration of ZA. This is supported by the lack of change in comparison to pre-administration samples for all post-administration sampling.

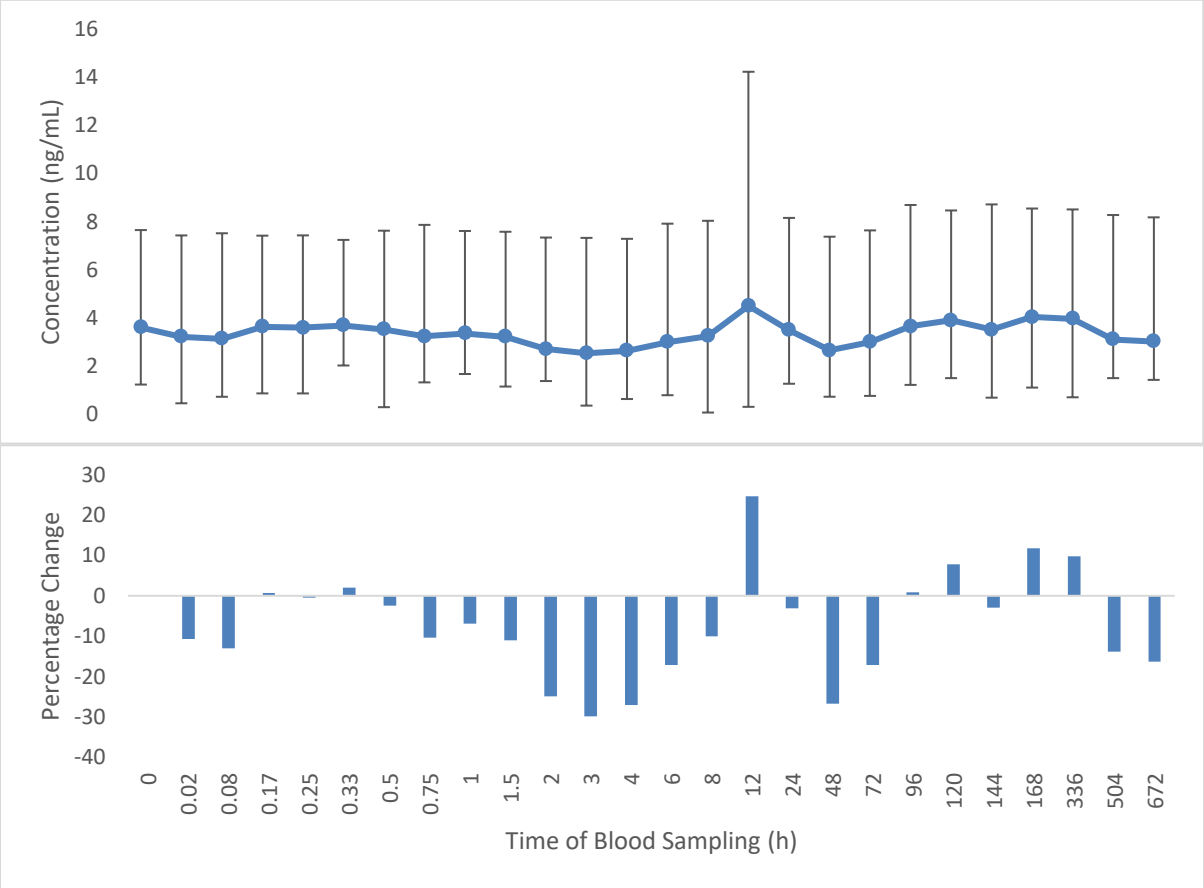

Figure 5.10: Average concentration of plasma 18-HEPE following ZA administration. Panel A showing the mean concentration and vertical bars representing the range; panel B showing the mean percentage change (n=8).

Other lipids and corticosteroid biomarkers (figures in appendices):

The other lipid and corticosteroid biomarkers were either not detected or did not show a consistent pattern to aid in detecting the presence of a ZA administration. 12-HETE showed relatively consistent concentrations throughout with no distinct increasing or decreasing levels in comparison to time 0 as seen in appendices Figure A14. Similarly for 13-HDHA and 17-HDHA, due to their isomerism, separation wasn't possible but for the combined response, the concentrations never exceeded 5 ng/mL as seen in appendices Figures A15. There is the

possibility of these two markers becoming ERCs given the consistency. 15-HEDE showed some decreasing levels up to 6 days (144 h) post-administration however, at 14-21 days (336 – 504 h) post-administration, this returned to basal before being slightly increased by 37% at 28 days (672 h) post-administration in comparison to time 0 as seen in Figure A16.

Throughout the ZA administration period, 15-HETE showed consistent peak areas as seen in appendices figure A17 however, at 7 to 14 days (168- 336 h) post-administration, increasing levels of 26% and 23% in comparison to time 0 was seen. Whereas 5-HETE showed some increasing levels at 2 hours and 28 days (672 h) post-administration (figure A18). Unfortunately, this was not in a pattern that could aid in the determination of ZA administration. Therefore, the ratio of 5(S):15(S)-HETE (as seen in appendices figure A19) whilst detectable, did not show an increasing or decreasing trend. However, this stability of the ratio could aid in the differentiation between a TA and ZA administration. The area for 9-HOTrE could not be quantified given the R^2 value being less than ideal. In appendices figure A20, 9-HOTrE has a large increase very early in the administration before decreasing in comparison to time 0. Unfortunately, having the large increase at the beginning of the administration, 9-HOTrE would not be a good biomarker especially since the aim is to indirectly extend the time of detection for ZA. AA was also detectable throughout the administration with a general increase of concentration until 5 days (120 h) post-administration before returning to basal levels according to time 0 as seen in appendices figure A21. There is however notable variation in the concentrations which can be attributed to the estimation of these concentrations. Prostaglandin D_2 (PGD_2) also showed some relative consistency before increasing at 28 days (672 h) post-administration as seen in appendices figure A22. The biomarker of $PGF_{2\alpha}$, did not show a large increase then decrease in the later time points as seen in appendices figure A23. Whilst during the ZA administration, there is some evidence of an increase and decrease between 6 to 28 days (144 – 672 h) post-administration. This differentiation could also potentially be useful to identify the difference between a TA and a ZA administration.

5.4.2 Semi-Targeted Results

A semi-targeted screen utilising the Shimadzu lipid mediator package was completed on the ZA administration samples following the method as set out in chapter 2.2.8.2. Following this method, the compounds in the package that showed change in comparison to time 0 throughout the ZA administration were 11-HEDE, 13-HODE, 14,15-DiHETE, 20-HDHA, 4-HDHA, 9-HEPE, 9-HETE, 9-HODE, DHA, and EPA. The integrated peak area for these markers were plotted to observe the change.

11-HEDE showed evidence of a decrease until 6 days (144 h) post-administration before increasing by 36% at 28 days (672 h) post-administration (figure A24 in appendices). For 13-HODE (figure 5.13), 14,15-DiHETE (figure A25) and 9-HODE (figure A26), these biomarkers displayed consistent peak areas not changing by more than 20% compared to time 0 until 28 days (672 h) post-administration where all biomarkers showed an increase in area. For 13-HODE (figure 5.13), this was a 27% increase, for 14,15-DiHETE (figure A25), the increase was 48% and for 9-HODE (figure A26), the increase was 28%. For 20-HDHA (figure A27), there is the presence of a slight increase 10 minutes post-administration where a 33% change was seen however, this returns to basal relatively quickly before decreasing between 3 hours to 6 hours post-administration. After 6 hours post-administration, the area once again increases until 7 days (168 h) post-administration where an increase of 46% is seen before a sharp decrease between 38-32% compared to time 0 between 21-28 days (504 – 672 h) post-administration respectively.

With DHA and EPA, as previously mentioned, given the position of these biomarkers in the respective lipid cascades, the presence and detection were expected. For DHA, the area was increased compared to time 0 from 1-minute post-administration to 327% at 6 hours post administration. There was a stable increased until 7 days (168 h) post-administration where a 37% decrease compared to time 0 was observed. This remained decreased of 29-35% until 28 days (672 h) post-administration as seen in Figure 5.11. EPA exhibited a similar pattern to DHA with an increase from 10 minutes at 140% compared to time 0. The integrated peak area continuously increased to the maximum at 6 hours post-administration of 367% compared to time 0 and remained increased until 7 days (168 h) post administration. However, unlike DHA,

at 14 days post-administration, the levels of EPA returned to basal levels as seen in Figure 5.12.

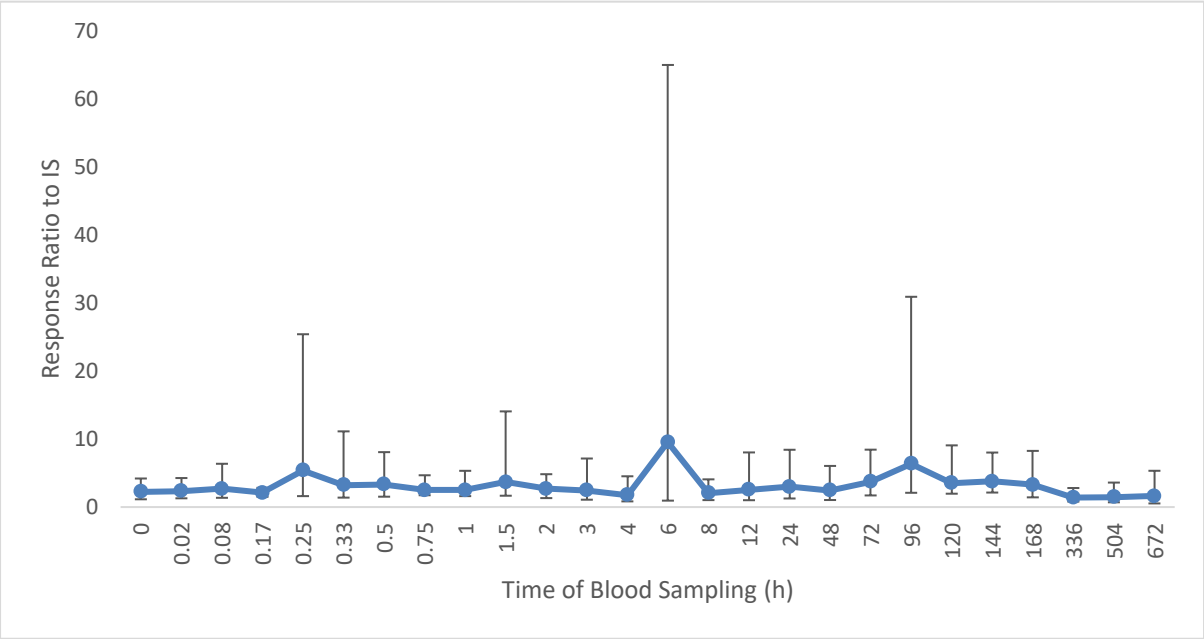

Figure 5.11: Average integrated peak area with vertical bars representing the range for plasma DHA with ZA administration (n=8).

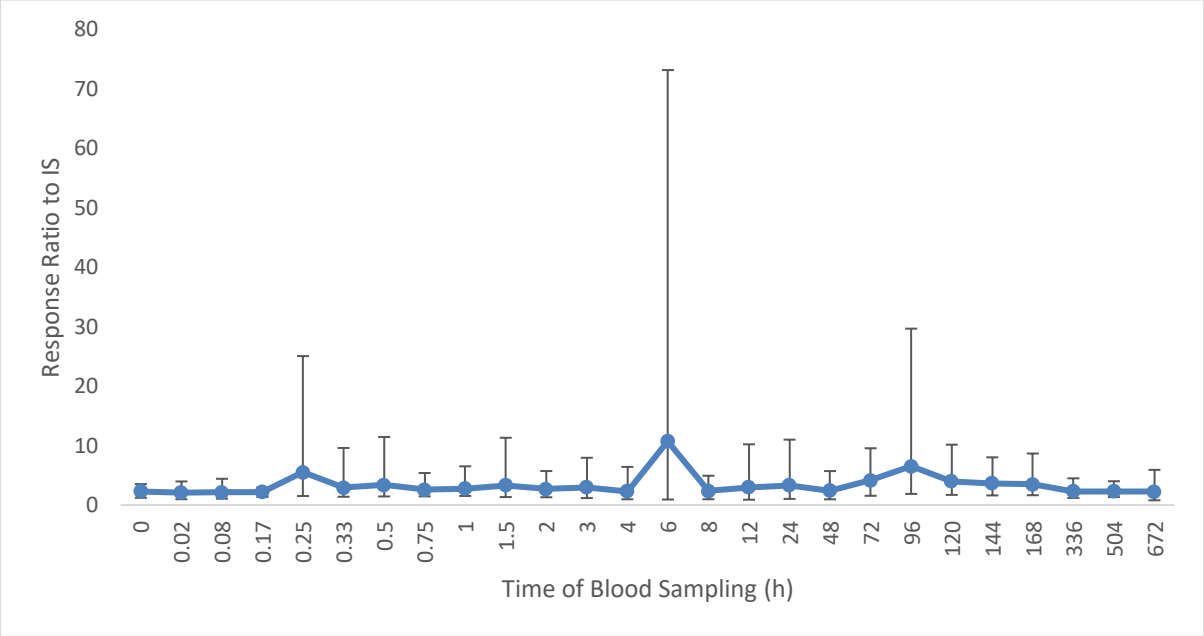

Figure 5.12: Average integrated peak area with vertical bars representing the range for plasma EPA with ZA administration (n=8).

The markers of 4-HDHA (figure A28), 9-HEPE (figure A29) and 9-HETE (figure A30) were detectable but there was no distinct increase or decrease seen. Therefore, these biomarkers could be considered as ERCs for future ratios with other lipid biomarkers.

5.4.3 Non-targeted Results

Untargeted analysis was completed for certain time points throughout the ZA administration. The time points chosen for ZA were 3-, 14- and 28-days (72, 336 and 672 h) post administration for Gelding A, B and C. The workflow utilised is detailed in chapter 2.2.8.3 Non-Targeted Screening Data Processing. From the positive acquisition unfortunately, no useful biomarkers were identified through the untargeted screening. For negative acquisition however, the lipid of 13-HODE was identified having change 14 days (336 h) post administration for Gelding A and in 28 days (672 h) post-administration for Gelding A, B and C. The identification of 13-HODE from the untargeted workflow provides verification of the results obtained from the semi-targeted workflow having also identified 13-HODE as a biomarker displaying change throughout the administration as seen in Figure 5.13 (for individual profiles, refer to appendices figure A31 to figure A33). No other lipid biomarker was detected through the untargeted analysis.

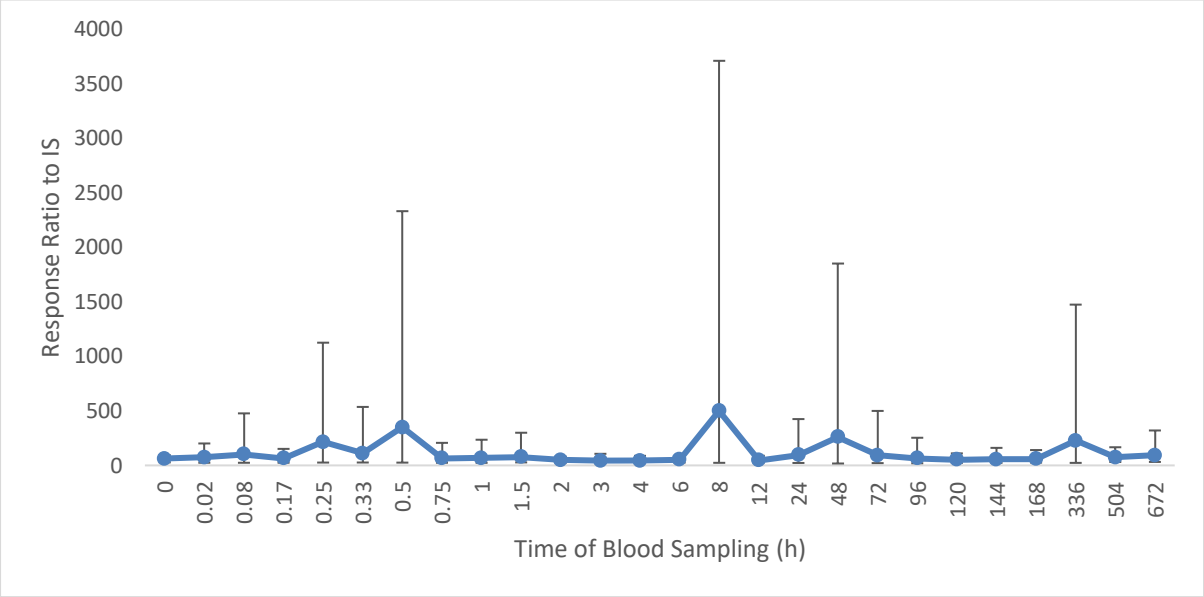

Figure 5.13: Average integrated peak area of plasma 13-HODE with vertical bars representing the range during a ZA administration (n=8).

5.4.4 Individual Reference Limits

Unfortunately, the ZA administrations only had one sample collected pre-administration, therefore longitudinal profiling could not be performed. As stated previously, the profiles require the use of at least 3 samples to be able to have the profile weighted towards the individual horse rather the population.

5.5 Results and Discussion from Tiludronic Acid Administration

5.5.1 Targeted Results

5.5.1.1 Tiludronic Acid Quantification

TA was administered to eight horses, (four males and four geldings). Plasma concentrations of TA in these horses are shown in figure 5.14.

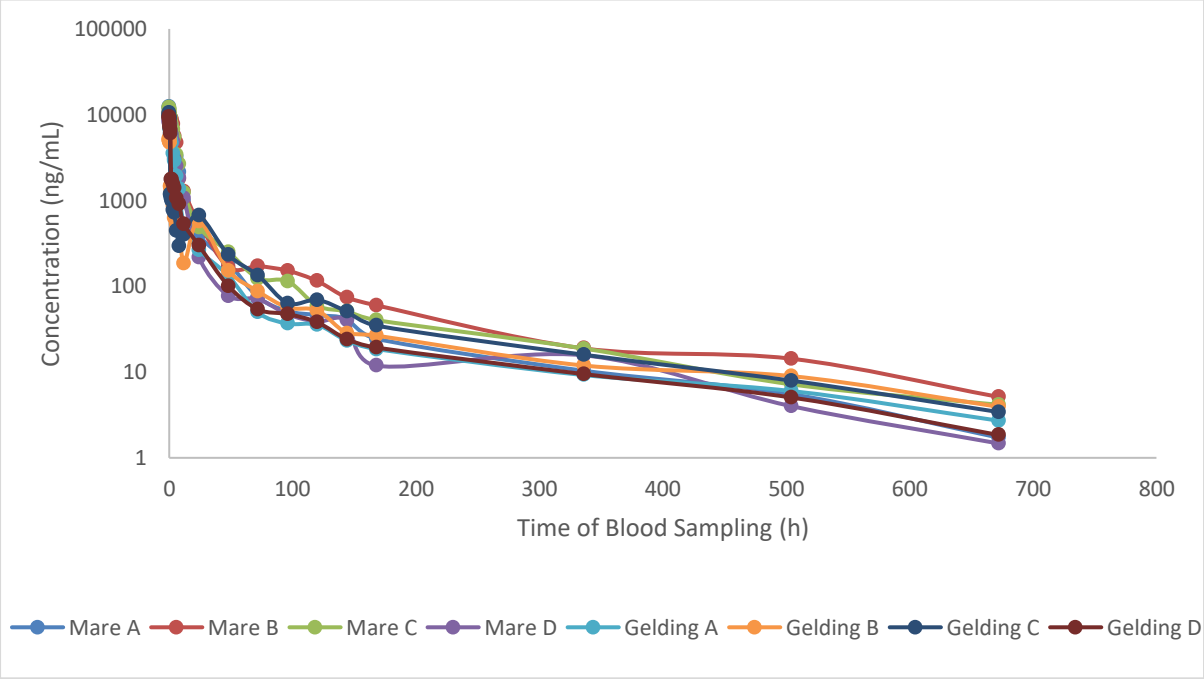

Figure 5.14: Elimination of TA in 8 administered horses using a log y-scale.

As seen in Figure 5.14, the elimination of TA for all eight horses. All eight horses displayed a reduction in plasma TA after the maximum concentration at 1-minute (0.02 h) post-administration (post full 30-minute (0.50 h) IV infusion). The highest concentration was estimated to be 12,500 ng/mL from Mare A with the lowest maximum concentration being estimated at 5,373 ng/mL from Gelding B illustrating large inter-individual variation of TA. The inter-individual variation could potentially be due to the slight deviation from the timing of collection (i.e. a minute or two later than 1-minute (0.02 h) post-infusion due to multiple horses being sampled at the same time) or incomplete mixing of the drug with the saline prior to administration into the horse. It was confirmed that all delivery was via catheter, so it was unlikely some of the drug was delivered into the subcutaneous tissue. Figure 5.14 shows a rapid decline in the TA concentration over the first 2 days post-administration. Figure 5.15 removes the high concentrations obtained after initial administration to show that TA is detected and quantified at the LOQ of 1 ng/mL at 28 days (672 h) post-administration.

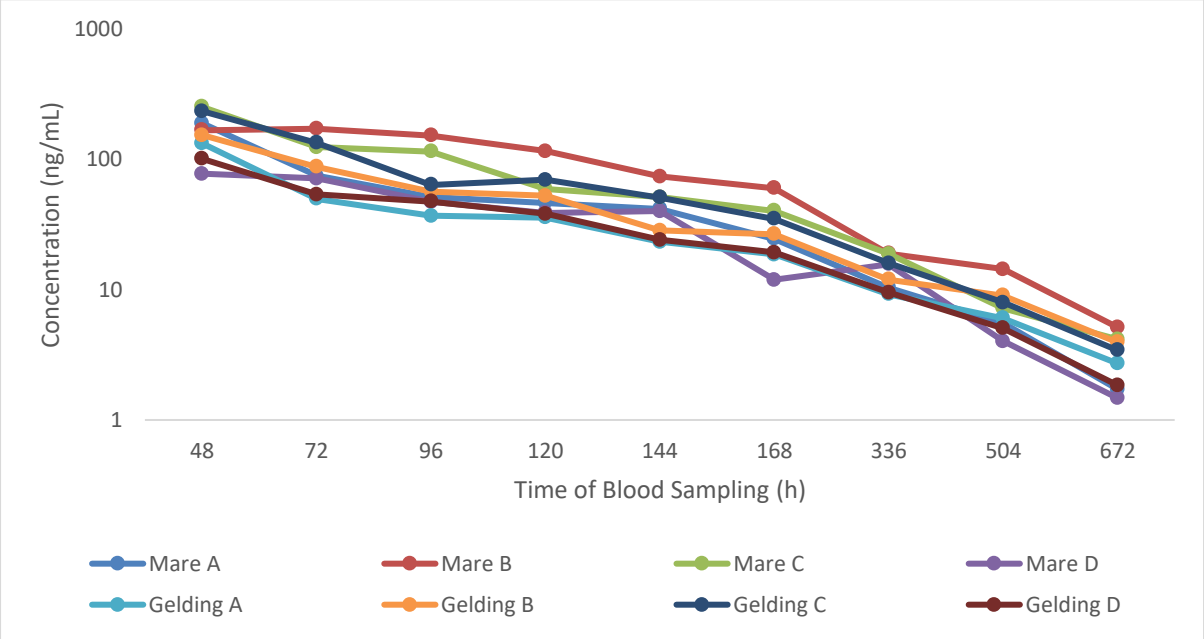

Figure 5.15: Elimination of TA in 8 administered horses using a log y-scale – tail end of the elimination curve

According to Figure 5.15, at 28 days (672 h), TA is still able to be quantified above the LOQ of 1 ng/mL. These results are consistent with those presented by Popot *et al*[89], where concentrations of TA were still detectable 15-days post-administration at less than 10 ng/mL.

Similarly, another study conducted by Popot *et al.*[126] showed plasma concentrations down to 2.5 ng/mL up to 30 days post-administration. With these results being consistent, there is the possibility of TA being detectable for a much longer period of time as seen in the study conducted by Riggs *et al*[121]. Concentrations of TA in Riggs' study was detectable in equine plasma up to 3 years post-administration. Whilst this study was only completed to 28 days (672 h) post-administration, there is still the evidence of a very long-detection time for TA. The concentrations at 2 years post-administration were still above the LOQ however, at 3-years post-administration, concentrations were above the LOQ for the 500 mg administration of TA whilst concentration was below LOQ but above LOD for the two horses that received 100 mg. The major disadvantage of this administration is the lack of sampling post 28 days (672 h) with the exact time of TA falling below LOQ unknown, it is likely that this may not occur for an extended period of time.

With this extended time period but low concentrations, it is needed for a complementary form of screening of lipid and corticosteroid biomarkers. Following analysis of the biomarkers, the following biomarkers showed notable change: Prostaglandin $F_{2\alpha}$, 5(S)-HETE/15(S)-HETE ratio, OEA and 18-HEPE. The biomarkers that remained static include AEA and HC/C. Throughout the course of the TA administration study; 7 lipids and 2 corticosteroids were not detected. These were: 11-dehydrothromboxane B_2, 6-keto prostaglandin $F_{1\alpha}$, leukotriene B_4, prostaglandin E_2, prostaglandin D_2, leukotriene D_4, leukotriene E_4, 18-hydroxycortisol and 18-oxocortisol. A major limitation of this study was the lack of placebo treated (control) horses. Therefore, it cannot be discerned in the biomarker work whether the effect was from TA and/or from external environmental factors. Additionally, all horses that were administered with ZA 12 months prior, were all administered with TA during this administration. Therefore, with the lack of placebo horses and randomisation from the ZA administration, it cannot be known whether the previous ZA administration had any long-lasting effects that may have affected the TA administration.

5.5.1.2 Lipid and corticosteroid biomarkers

Prostaglandin $F_{2\alpha}$:

Prostaglandin $F_{2\alpha}$ demonstrated the most interesting plasma profile following TA administration. Due to the inability to quantify the concentration, the integrated peak area was used to monitor the change occurring using the corresponding labelled internal standard ($PGF_{2\alpha}$-D_4). Throughout this administration as seen in Figure 5.16 (top), there was a consistent but slow increase from time of administration however, between 5 to 6 days (120 – 144 h) post-administration there is a very sharp increase. This biomarker then proceeds to have a rapid decrease between 14 days and 21 days (336 – 504 h) post-administration. At the 28-day (672 h) post-administration mark, there is an inconsistent increase/decrease for this biomarker as that depends on the individual horse. Using the average percentage change in Figure 5.16 (bottom), at 6 days (144 h) post-administration, there is a percentage change of 142% which is the largest increase throughout the administration but at 28 days (672 h) post-administration, there is a 66% decrease. Table 5.8 highlights the fold change (FC) and p-value for the time points of 5, 6, 7, 14-, 21- and 28 days (120 – 672 h) in comparison to pre-administration samples.

Table 5-8: Fold change and p-values for Prostaglandin $F_{2\alpha}$ during the TA administration compared to pre-administration samples.

Time point	Fold-change	p-value
5 days (120 h)	1.5	0.0050
6 days (144 h)	2.7	0.00030
7 days (168 h)	2.0	0.0020
14 days (336 h)	0.0020	0.99
21 days (504 h)	-0.49	0.23
28 days (672 h)	-0.48	0.23

With respect to table 5.8, the time points of 5, 6 and 7 days (120 – 168 h) showed the most change in comparison to time 0 however, the decreased time points did not fulfil the criteria for significance. Nonetheless this large change seen between 7 to 28 days (168 – 672 h) post-administration is extremely advantageous given the longevity of the detection of TA itself. Therefore, PGF_{2a} will be an important biomarker to monitor for a non-nitrogenous bisphosphonate.

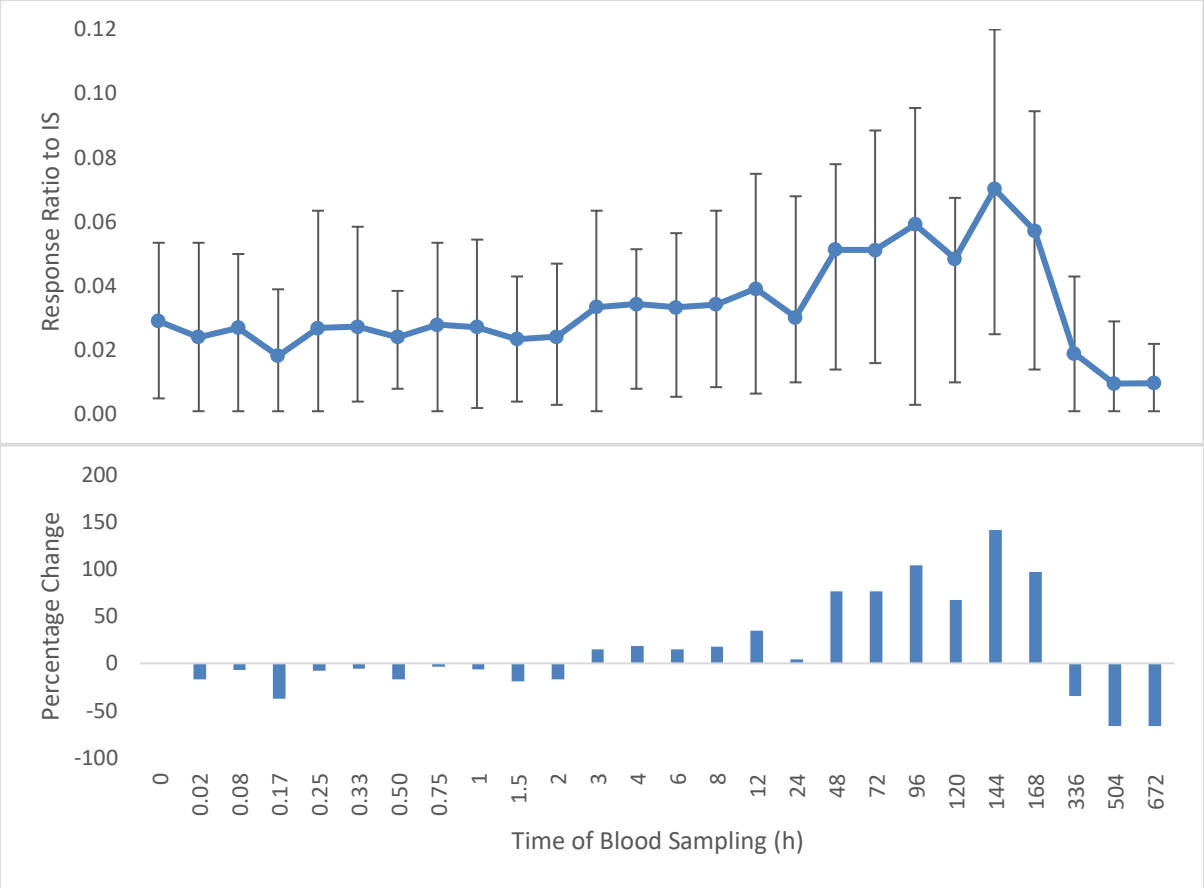

Figure 5.16: Average peak area response for plasma $PGF_{2\alpha}$ profile following TA administration. Top panel showing the integrated peak area with the vertical bars representing the range; bottom panel showing the average percentage change (n=8).

The biological relevance of $PGF_{2\alpha}$ was hypothesised to be relevant to the anti-inflammatory activity that a non-nitrogenous bisphosphonate display. The biological relevance is currently only known in humans. The anti-inflammatory activity is caused by the inhibition of the release of inflammatory mediators from activated macrophages (white blood cells at the site of infection)[133]. These inflammatory mediators include those of Interleukin-1 (IL-1)[134]. In

humans, the evidence of IL-1 stimulated chondrocytes (cells responsible for cartilage formation)[135], may be related to the synthesis of $PGF_{2\alpha}$ which may explain the sharp increase seen at 6 days post-administration for this biomarker.

15(S)-HETE:

The area ratio of detected 15(S)-HETE to its labelled internal standard was used to monitor a TA administration as the concentrations were unquantifiable. As TA is quantifiable for an extended period of time (28 days/672 h), the use of this biomarker can complement direct detection as seen 7- and 28-days (168 and 672 h) post-administration with a decrease of 14-64% compared to time 0 as seen in Figure 5.17.

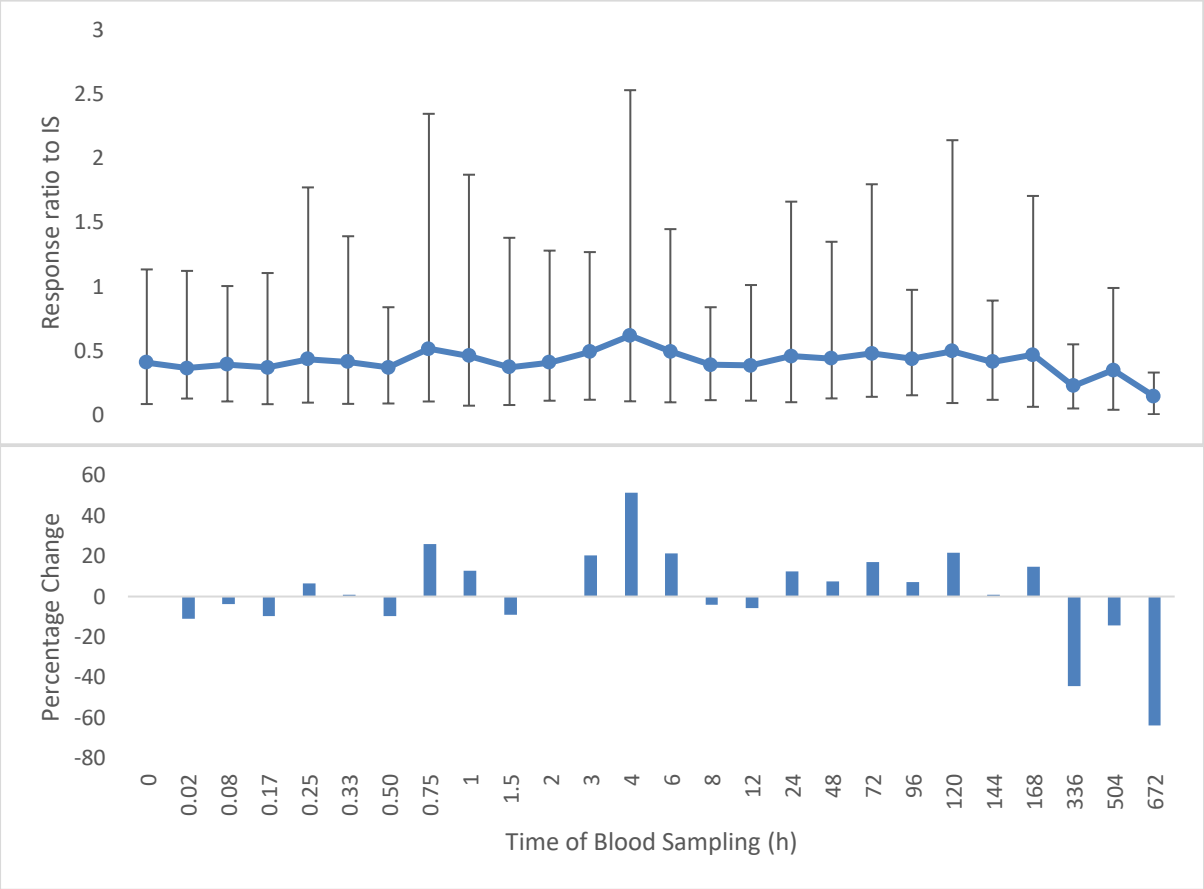

Figure 5.17: Average peak area response for plasma 15(S)-HETE profile following TA administration. Top panel showing the integrated peak area with the vertical bars representing the range; bottom panel showing the average percentage change (n=8).

5(S)-HETE:

For 5(S)-HETE, similar to 15(S)-HETE, integrated area was used due to non-quantifiable levels. Using the average area as seen in Figure 5.18 compared to time zero, there is a general decreasing trend towards the end of the study period.

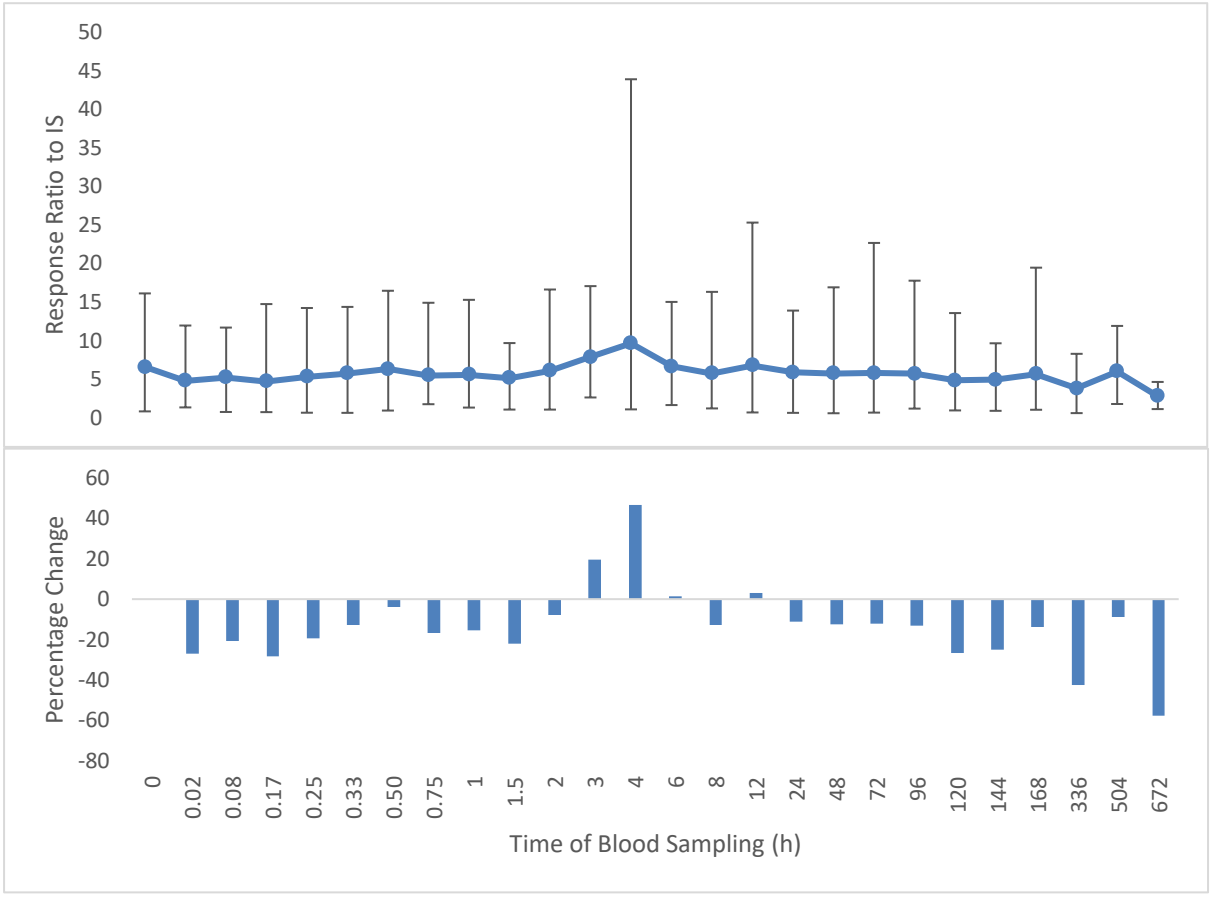

Figure 5.18: Average peak area response for plasma 5(S)-HETE profile following TA administration. Top panel showing the integrated peak area with the vertical bars representing the range; bottom panel showing the average percentage change (n=8).

5(S)-HETE : 15(S)-HETE Ratio:

The 5(S)-HETE to 15(S)-HETE ratio was used to observe whether a pattern can be seen normalising to another biomarker. The area ratio of the detected peak area of the two biomarkers to their respective labelled internal standards were used since the concentrations for both were not quantifiable[136]. The ratio appeared to be consistent to pre-administration samples up to 7 days post-administration with a percentage change not higher than 35% as

seen in Figure 5.19. Between 21 to 28 days (504 – 672 h) post-administration, an increase was observed of between 120 and 132% with these two time points showing the most change in comparison to pre-administration samples (Figure 5.19 and Table 5.9).

Table 5-9: Fold change and p-values for 5(S)-HETE : 15(S)-HETE ratio during the TA administration

Time point	Fold change	p-value
21 days (504 h)	1.5	0.0011
28 days (672 h)	1.6	0.00075

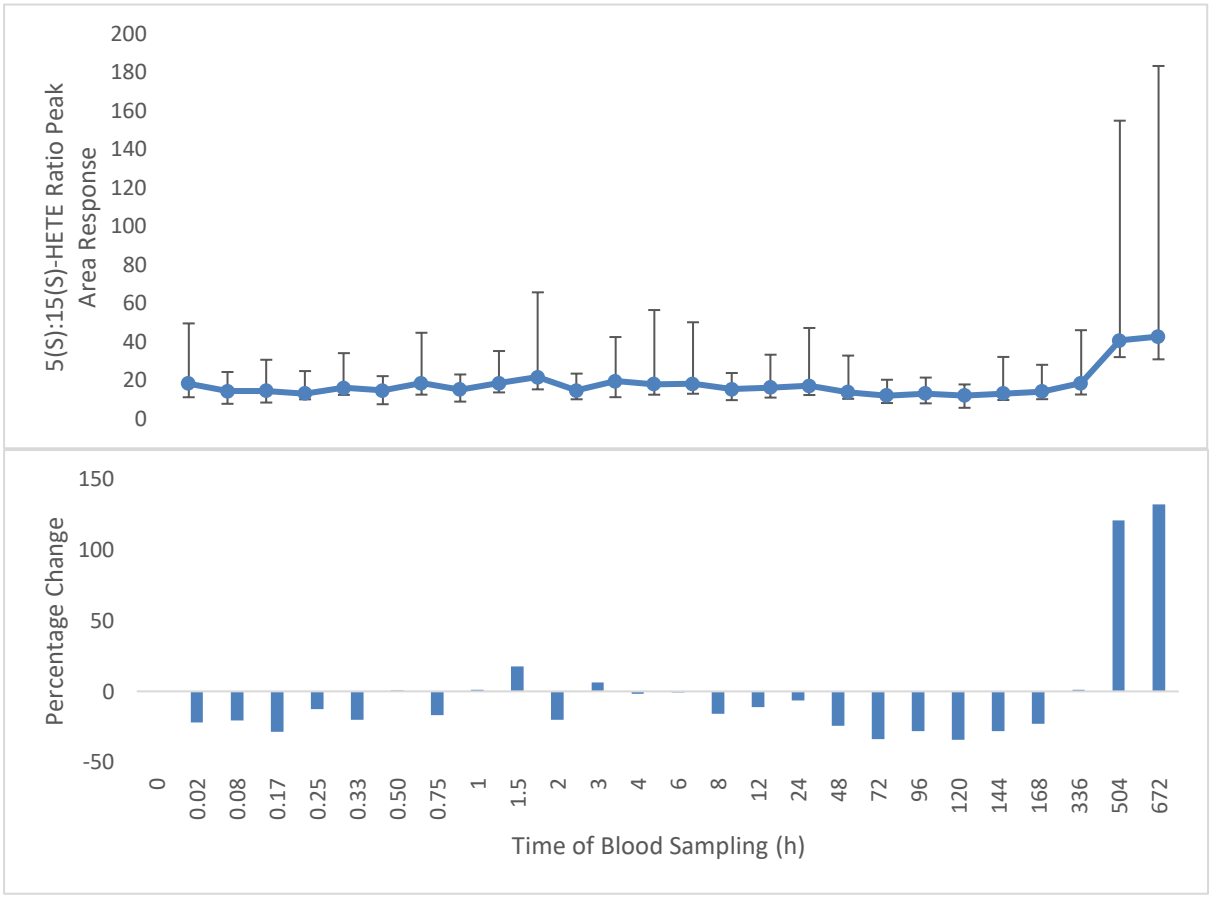

Figure 5.19: Average peak area ratio of 5(S)-HETE to 15(S)-HETE following TA administration. Top panel showing the integrated peak area ratio with vertical bars representing the change; bottom panel showing the average percentage change (n=8).

Little is known about the biological relevance of both 5(S)-HETE and 15(S)-HETE in the equine system nor in humans under the influence of BPs. However, in relation to the AA cascade, 15(S)-HETE is derived from the 15-LOX enzyme with an intermediate of 15(S)-HPETE. With a decrease of 15(S)-HETE, it can be hypothesised that the 15-LOX enzyme isn't being produced to convert AA into 15(S)-HPETE and consequently into 15(S)-HETE. Similarly for 5(S)-HETE where the integrated peak area was also decreased towards the end of the study. It also be hypothesised that the 5-LOX enzyme isn't being produced as readily therefore not converting 5(S)-HPETE (the 5(S)-HETE intermediate) consequently, decreasing the amount of 5(S)-HETE present in the plasma. The lack of leukotrienes being detected in the plasma also supports the theory of a lack of 5-LOX enzyme as 5(S)-HPETE can hydrolyse to become leukotriene A4 and subsequently LTB_4, LTC_4, LTD_4 and LTE_4. The latter leukotrienes were not detectable throughout the entire administration. A possible cause is the analgesic effects BPs have on the equine system causing a later reaction especially as TA is detectable for a longer period of time. The delayed reaction is subsequently causing 5(S)-HETE and 15(S)-HETE to increase later than at the beginning stages of the administration.

AEA:

AEA was one of the few biomarkers monitored throughout the administration that was quantifiable however, estimated concentrations were considered low (less than 1 ng/mL). With the estimated concentrations of AEA, there wasn't any distinct pattern and with a few estimated concentrations lower than the LOQ, the peak area was monitored as seen in figure 5.20. But with its consistency in being detected it was considered a possible endogenous reference compound (ERC).

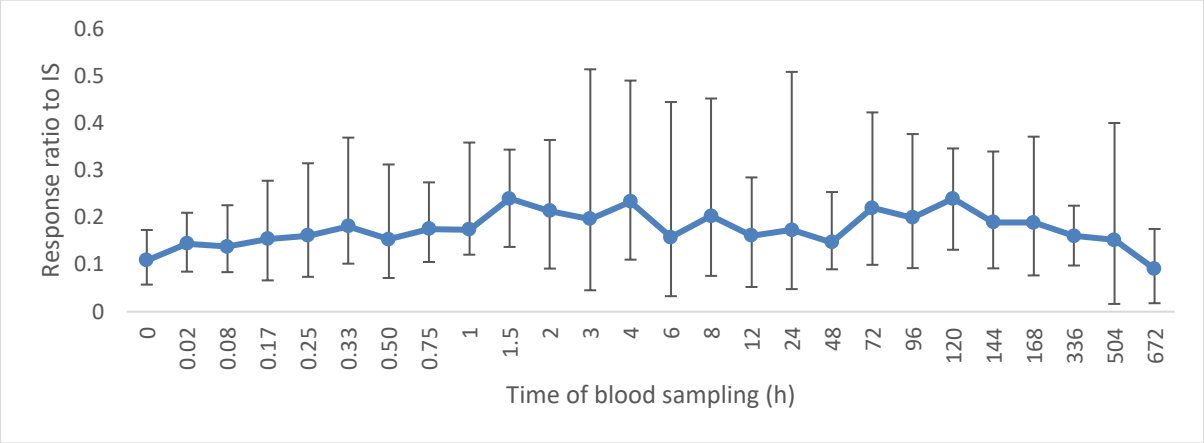

Figure 5.20: Average integrated peak area with vertical bars representing the range for plasma AEA with a TA administration (n=8)

OEA:

The biomarker of OEA was quantifiable throughout the administration period with concentrations of 4 ng/mL. Looking at Figure 5.21, there is consistently an increase with only the 2- and 28 days (48 – 672 h) post-administration mark being decreased. At 2 days (48 h) post-administration, it is decreased by 8.27% whilst at 28 days (672 h) post-administration, the decrease was at 33%. However, at 28 days post-administration, the FC was -0.61 which doesn't fulfil the criteria of being less than 0.67 for a significant value[103]. With the implementation of the proposed PRL of 5.6 ng/mL and 0.21 ng/mL from chapter 2.4.2, it can be seen that there are time points (8 h, 1 day (24 h), 3 – 14 days (72 – 336 h) post administration) that exceed the upper PRL. These samples would ideally be flagged as abnormal for further investigation also indicating that the PRLs are appropriately set. This is due to the presentation of the effect of TA on the biomarkers and the effects can be seen a few days later than the initial administration.

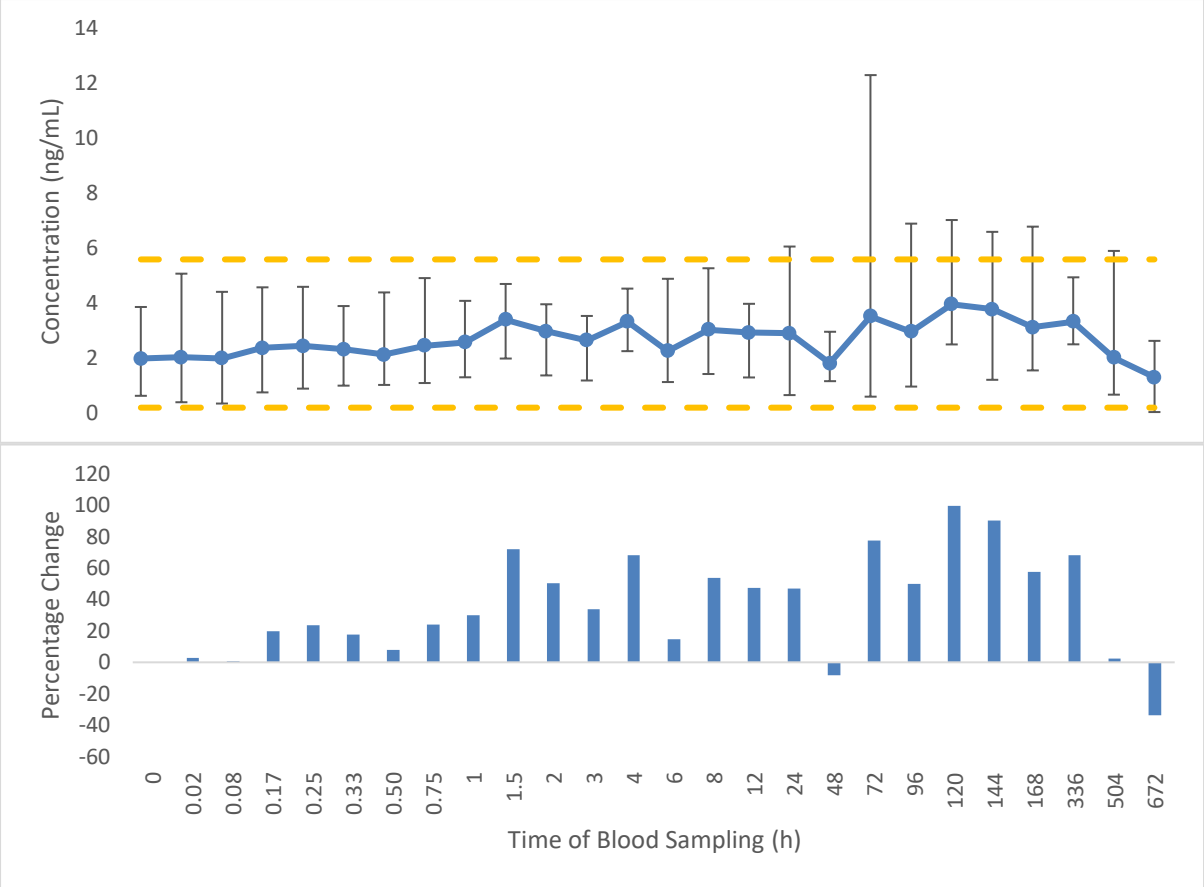

Figure 5.21: Average concentration of plasma OEA following TA administration. Top panel showing the concentration with vertical bars representing the range and yellow dotted line representing the PRL as proposed from Chapter 2 (proposed upper = 5.6 ng/mL and lower = 0.2 ng/mL); bottom panel showing the average percentage change (n=8).

Hydrocortisone/cortisone ratio:

The hydrocortisone to cortisone (HC/C) ratio has been demonstrated to be useful for monitoring the administration of exogenous drugs. However, in the presence of TA, this marker has not been as useful given there is limited increase or decrease in comparison to time 0 as seen in Figure 5.22. As seen in Figure 5.22, the percentage change for the earlier time points is greater than 20% however, for the later time points where a TA administration would benefit, the percentage change is not as evident. This consistency in concentration, however, can be useful in differentiating between a TA administration and a ZA administration. Sampling for horses that occurred once a day was completed at approximately 8 am each day, therefore diurnal variation was not accounted for in this study.

With hydrocortisone being elevated in the presence of stress, it should be noted that the horses used for this administration are research horses where baseline hydrocortisone levels would not be as elevated as racehorses. This could be a contributing factor to the levels of HC/C not being as elevated.

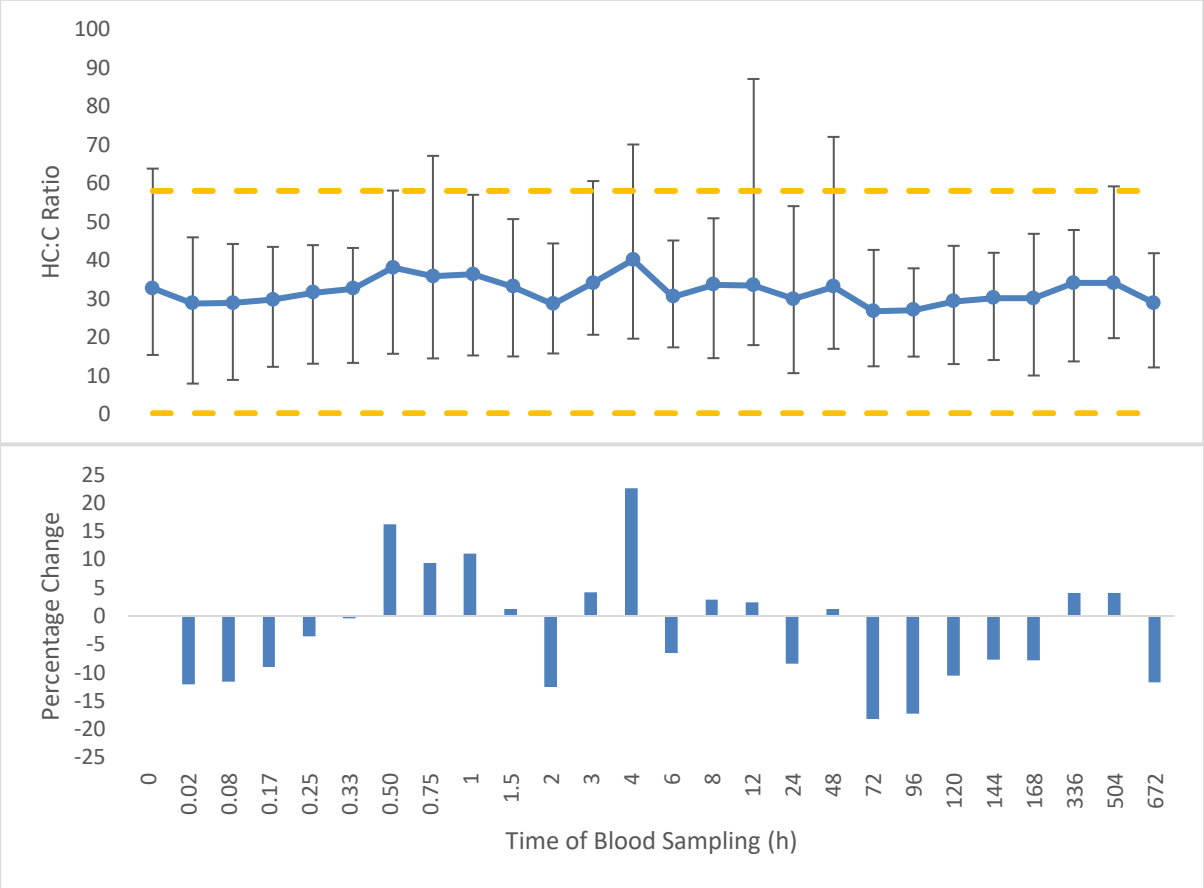

Figure 5.22: Average plasma HC/C concentration ratio following TA administration. Top panel showing the HC/C ratio with the vertical bars representing the range and the proposed PRL (yellow dotted lines with upper (58) and lower (0.24); bottom panel showing the average percentage change (n=8).

18-HEPE:

The concentration of 18-HEPE was relatively consistent with no distinct pattern throughout the administration period. At 14 days (336 h) post-administration however as seen in Figure 5.23, the concentration of 18-HEPE was decreased by 36% in comparison to time 0. This pattern was seen also in 21-28 days (504 – 672 h) post-administration with concentrations

being 26% and 55% decreased respectively. These two-time points, however, did not exhibit the most change in comparison to pre-administration samples.

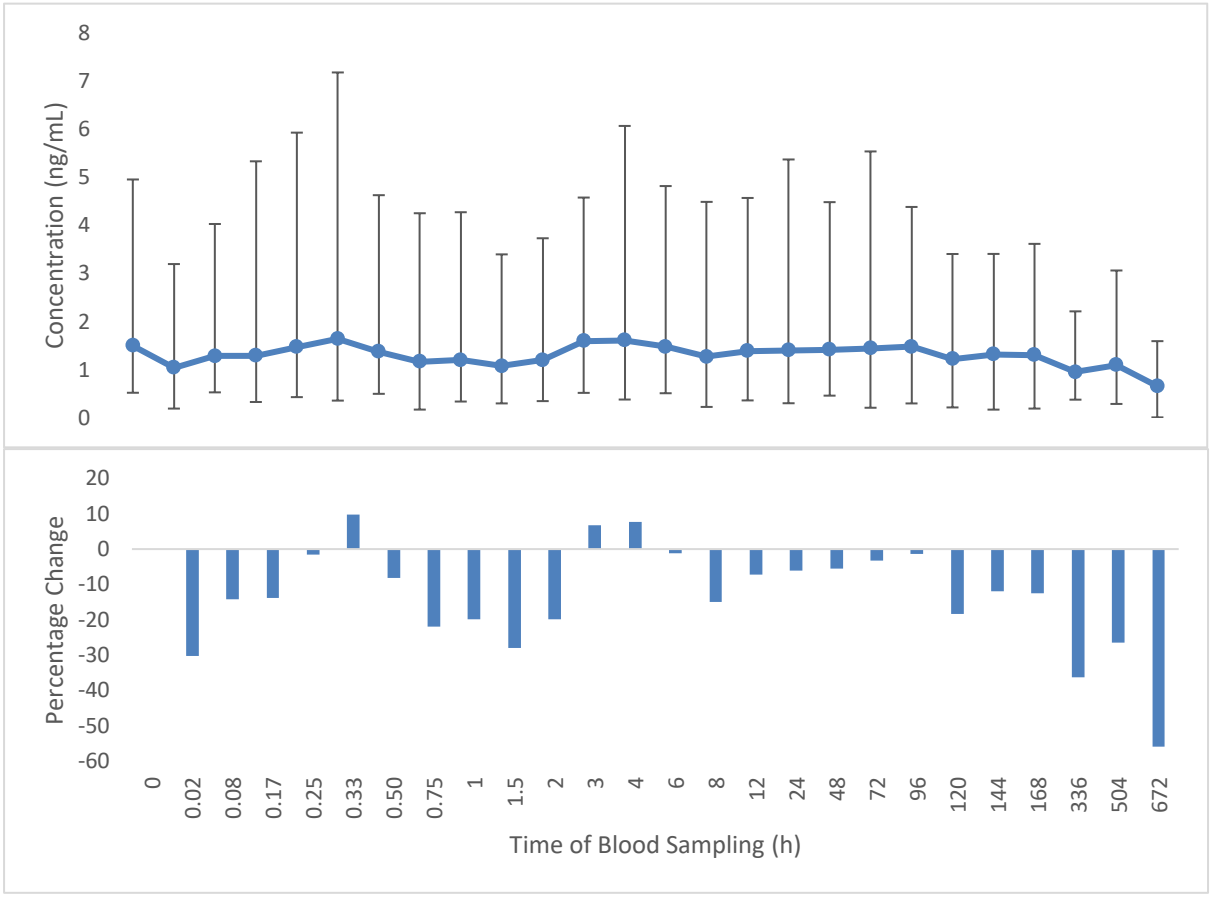

Figure 5.23: Average estimated concentration of plasma 18-HEPE following TA administration. Top panel showing the estimated concentration with the vertical bars representing the range; bottom panel showing the mean percentage change (n=8).

Arachidonic Acid (AA):

As the precursor to the eicosanoid cascade, the monitoring of AA was pertinent to observe whether change could be seen for this biomarker compared to the other lipids lower down the cascade. However, the concentrations observed were consistently higher than the highest calibrator of 200 ng/mL, therefore, integrated peak area was used to monitor this biomarker for consistency. As according to Figure 5.24, the average peak area for AA during a TA administration is consistent with no evidence of increasing levels. The greatest change was 35% at 2 days (48 h) post-administration with consistent decreasing levels until 21 days (504 h) post-administration as seen in Figure 5.24. The consistent detection of AA is to be expected

as it is the precursor to the eicosanoid cascade however, it is evident that having higher levels of AA doesn't constitute to lower levels of the lipids further down the cascade and vice versa.

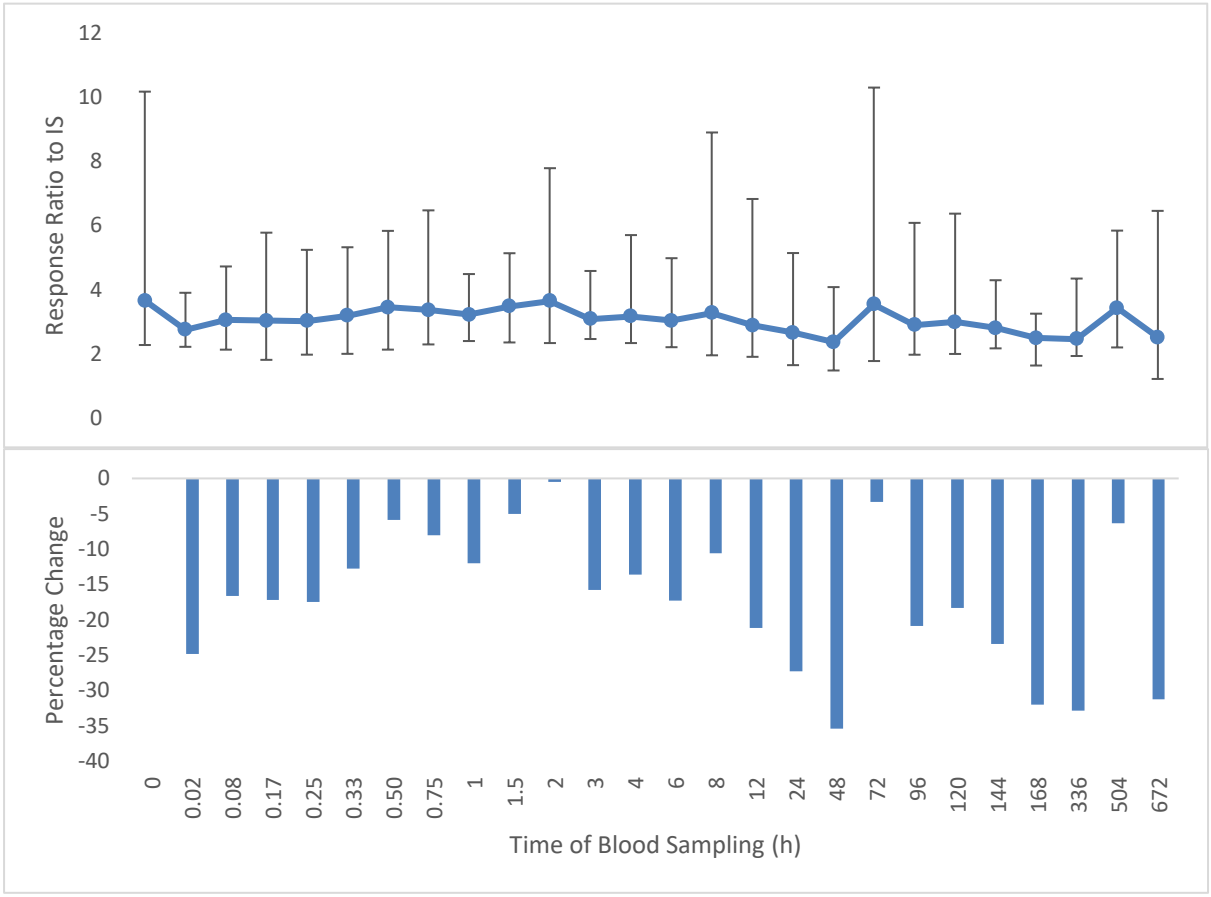

Figure 5.24: Average peak area response of plasma AA profile following TA administration. Top panel showing the average integrated peak area with vertical bars representing the range; bottom panel showing the mean percentage change (n=8).

Other lipid and corticosteroid markers (figures in appendices)

The area of 12-HETE was monitored due to quantification not being possible. There was no obvious pattern to the levels of 12-HETE (as seen in appendices figure A34). As seen however, between 7 to 14 days (168 – 336 h) post-administration, there was consistent decreasing levels from 22% to 66% change compared to time 0 as seen in appendices figure A34. For the estimated concentrations of 13-HDHA and 17-HDHA, it was hypothesised that both these isomers co-elute with each other therefore, these two were to be discussed together. As the concentrations were never greater than 10 ng/mL, these two markers are possible to be endogenous reference compounds. However, even with the consistent concentrations as

seen in appendices figure A35 top panel, at 28 days (672 h) post-administration, on average there is a decrease of 36% compared to time 0 as seen in appendices figure A35 bottom panel. Regarding 15-HEDE, the area was utilised as this marker was not quantifiable. Based on appendices figure A36 in the top panel, there is no consistent pattern with 15-HEDE however, it was always present throughout the administration period. Compared to time 0, 14-28 days (336 – 672 h) post-administration had a percentage change between 40-55% as seen in appendices figure A36 in the bottom panel. Similarly for 9-HOTrE, the area was utilised to monitor the effects of TA as the R^2 value of the calibration curve wasn't acceptable. As seen in appendices figure A37, at 3 hours post-administration, there is a consistent increase amongst all 8 horses however, there is then an erratic elimination pattern at the later time points. Unfortunately, this makes 9-HOTrE a difficult biomarker to monitor.

For the corticosteroid markers, estimated hydrocortisone concentrations did not exceed 180 ng/mL however, there is a consistent increase at 4 hours post-administration (as seen in appendices figure A38). Based on this elimination curve, there is no consistent change which makes hydrocortisone a difficult marker to use to identify a TA administration. Comparatively for cortisone, based on previous knowledge of cortisone, it still follows a consistent concentration of not being over 5 ng/mL as seen in appendices figure A39. There is a slight increasing and decreasing of levels but as the concentrations are very small, this is difficult to monitor. Therefore, cortisone is more useful as an endogenous reference compound as stated earlier for the hydrocortisone to cortisone ratio (HC/C). The last corticosteroid biomarker monitored that showed activity during a TA administration is 11-deoxycortisol. As seen in appendices figure A40 top panel, the area was once again monitored due to the inability to quantify but with this marker, there was at least one large increase early during the post-administration. On average, this was seen around between 15-90 minutes (0.25 – 1.5 h) post-administration (between 187% - 604% increase in comparison to time 0) as seen in appendices figure A40 bottom panel. However, as mentioned previously, the time points that are advantageous for detecting TA administration would be the later time points of 14-28 days (336 – 672 h) post-administration.

5.5.2 Semi-Targeted Results

A semi-targeted analysis of the lipids was performed utilising the Shimadzu lipid exact mass package containing 196 lipids and 18 internal standards with retention time matching for isomer assignment acquired in both positive and negative mode. Criteria was set for confirmation with chromatography peaks having a signal to noise baseline of three or more, retention time within 0.1 minutes of the retention time as determined by the method package and the acquired mass of the compound being within 5 ppm error of the theoretical mass. Compounds that fit the set criteria had the peak area plotted to determine if there was distinct activity for further analysis. The compounds that met these criterions for a TA administration were 10-HDHA, 11-HEDE, 13-HODE, 14-HDHA, 4-HDHA, 7-HDHA, 9-HEPE, 9-HODE, DHA, and EPA.

For 10-HDHA (figure A41), there was a consistent increase at 21 days (504 h) which was evident in all horses with an average of 23% increase compared to time 0 before a very sharp decrease at 28 days at an average of 82% compared to time 0. The presence of 14-HDHA (figure A42) was consistent however, there was no distinct pattern until 21 days (336 h) post-administration where an increase was seen at an average of 22% then a sharp decrease of 80% on average compared to time 0. Similarly for 7-HDHA (figure A43), there was a consistent decrease of the area compared to time 0 before a small increase at 21 days (504 h) post-administration of 13% before seeing a distinctive decrease of 78%.

Docosahexaenoic acid (DHA), similar to AA is the precursor to another chain of lipids in a separate cascade. DHA is an omega-3 fatty acid (compared to omega-6 for AA) in a similar manner to AA, is at the top of this part of the lipid cascade, it was hypothesised that there would be the presence of this compound in high amounts. Looking at the amounts of DHA present in equine plasma and using the internal standard of AA-D_8, there appears to be an increase at an average of 47% at 21 days (504 h) before an average 49% decrease at 28 days (672 h) post-administration as seen in Figure 5.25.

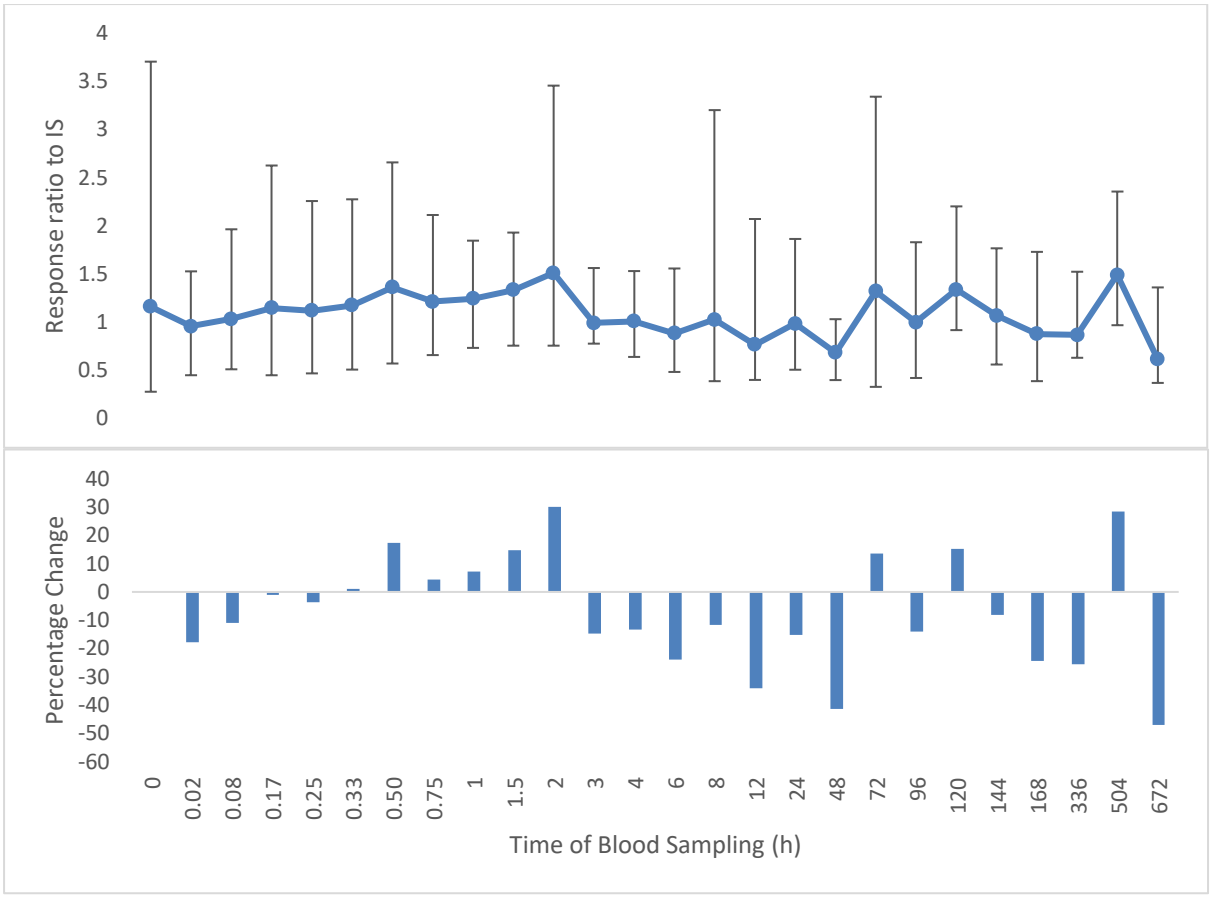

Figure 5.25: Averaged peak area response of plasma DHA following TA administration. Top panel showing the integrated peak area with the vertical bars representing the range; bottom panel showing the average percentage change (n=8).

Eicosapentaenoic acid (EPA) also an omega 3 fatty acid similar to DHA and AA was also present in the equine plasma samples which is to be expected given that it is also at the top of its relevant cascade. EPA appears to have a consistent decrease in area plotted against the internal standard of AA-D_8 in comparison to time 0. There is no evidence of increasing levels present as seen in Figure 5.26.

For 11-HEDE (figure A44), 13-HODE (figure A45), 4-HDHA (figure A46), 9-HEPE (figure A47) and 9-HODE (figure A48), decreasing levels was mainly observed in comparison to time 0 however, there was no distinct or consistent pattern for these markers.

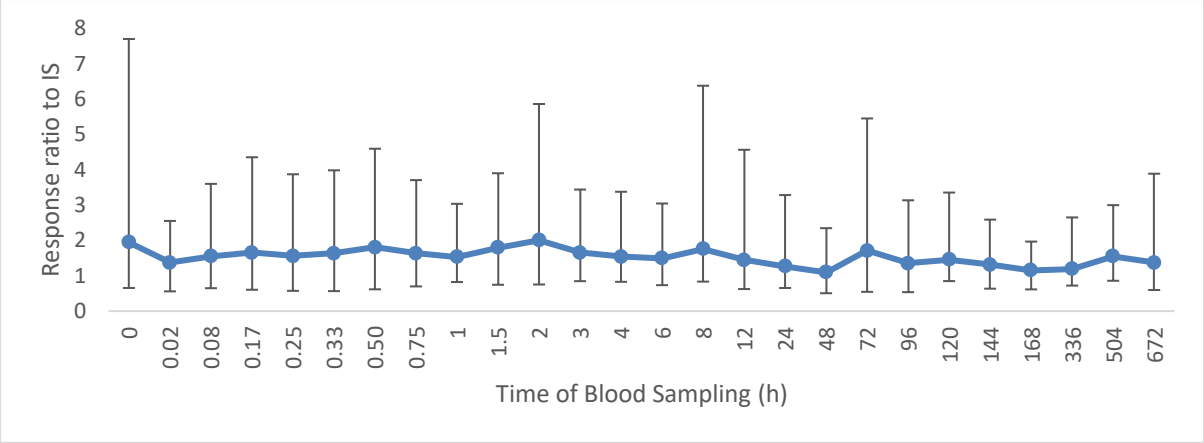

Figure 5.26: Average peak area response with vertical bars representing the range for plasma EPA with TA administration (n=8)

5.5.3 Non-targeted Results

As data was acquired through data independent acquisition on the LC-HRMS, there was the potential for untargeted analysis to be completed at certain time points along the sampling period. Time points were chosen based on compound maxima throughout the elimination curves and time points that were consistent with routine analysis. Therefore, 2 time points were chosen for untargeted analysis at 3- and 14-days post-administration for TA. The workflow utilised is detailed in chapter 2.2.8.3 Non-Targeted Screening Data Processing. From the positive and negative acquisition unfortunately, no useful biomarkers were identified through the untargeted screening.

5.5.4 Individual Reference Limits

As the administration of TA included 7 pre-administration samples, this allowed for the use of individual reference limits to profile the hydrocortisone/cortisone ratio. The profiles are calculated using the equations as stated in chapter 2.2.9. Figure 5.27 highlights an example profile of HC/C using Gelding D in the presence of TA.

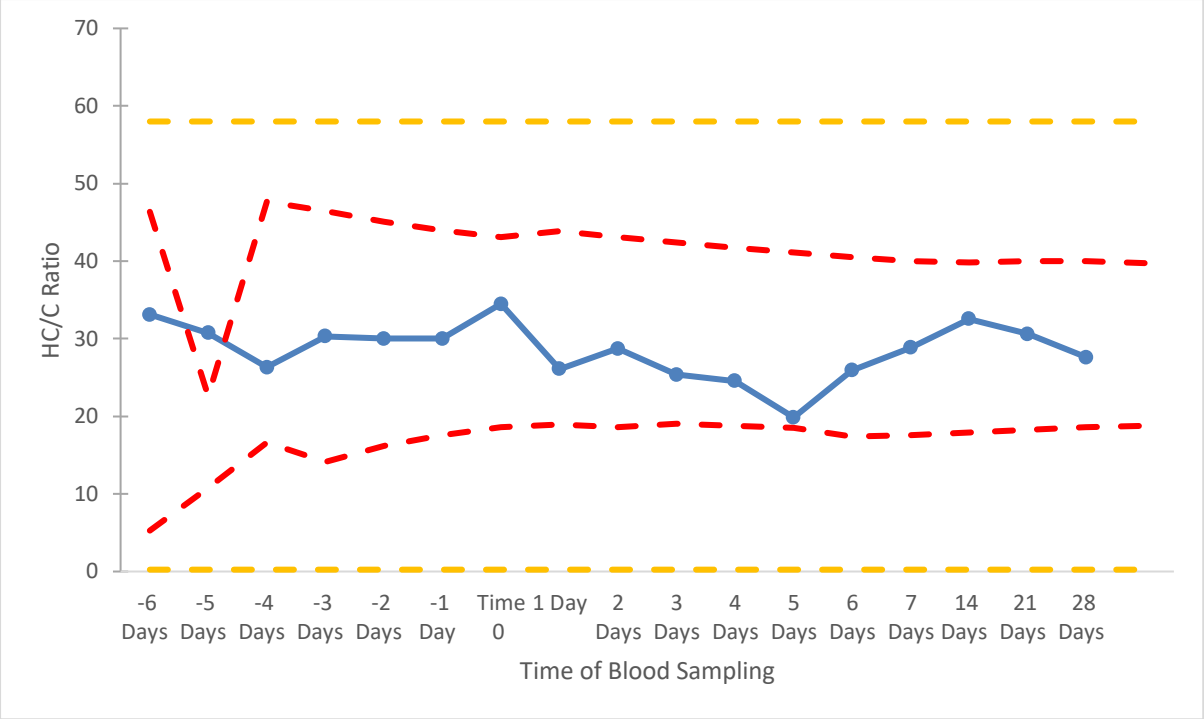

Figure 5.27: Intra-individual profile for the TA administration for Gelding D using the HC/C ratio (Legend: blue = HC/C ratio, red = individual upper and lower thresholds, yellow = proposed population upper and lower thresholds).

As seen in Figure 5.27, whilst the HC/C ratio doesn't exceed any population or individual reference limits, at 5 days post-administration, is it very close to the lower individual reference limit. At 5 days post-administration, the HC/C ratio is calculated to be 19.9 whilst the lower individual reference limit is calculated to be at 19 for that time point. This time point highlights how advantageous the use of IRLs is given that with the proposed population threshold, this sample would not be picked up as abnormal or one that is in the presence of an administration. The ratio exceeding the IRL at 5 days pre-administration isn't considered an abnormal value as the profile requires the use of at least 3 samples before the profile is more weighted towards the individual. With only 1 sample, the profile is still heavily weighted towards the population, therefore, the sample at 5 days pre-administration can be disregarded as abnormal.

For the profiles of the other 7 horses that underwent a TA administration, the profiles can be found in appendices figures A49 to A55.

5.6 Tiludronic and Zoledronic Acid administration comparisons

The biomarkers in the TA and ZA administrations were reviewed for differentiation between a nitrogenous and a non-nitrogenous BP administration. Table 5.10 highlights the biomarkers discussed previously which were able to complement the administration of TA as TA was detectable up to 28 days (672 h) post-administration. Table 5.11 highlights the biomarkers also discussed previously which indirectly extended the detection period of a ZA administration as ZA was only detectable up to 8 hours post-administration.

Table 5-10: Biomarkers that complemented the detection of TA.

Biomarker	Change observed	Time points post-administration
$PGF_{2\alpha}$	Increase	1 minute – 6 days (0.02 – 144 h)
	Decrease	14 – 28 days (336 – 672 h)
OEA	Increase	90 minutes – 14 days (1.5 – 504 h)
	Decrease	28 days (672 h)
18-HEPE	Decrease	14 – 28 days (336 – 672 h)
5(S)-HETE/15(S)-HETE ratio	Increase	21 – 28 days (504 – 672 h)

Table 5-11: Biomarkers indirectly extending the detection period of ZA.

Biomarker	Change observed	Time points post-administration
HC	Increase	21 – 28 days (504 – 672 h)
OEA	Increase	3 hours – 7 days (336 h)
	Decrease	14 – 21 days (336 – 672 h)
HC/C	Increase	21 days (504 h)

With each administration, the different biomarkers have the potential to either complemented or indirectly extended the detection period pending future population studies. Given the lack of placebo treated horses for these administrations, it is difficult to

ascertain whether the changes seen were due to external factors (e.g. diet, disease, exercise or from the administered drug itself. For TA, whilst the parent drug itself was detectable up to 28 days (672 h) post-administration, it must be noted that as indicated in literature by Riggs *et al.* the detection of TA over extended periods of time can be deemed erratic. Therefore, the detection of $PGF_{2\alpha}$ (increase and rapid decrease), 18-HEPE (decrease) and the 5(S)/15(S)-HETE (increase) provided evidence of effect up to 28 days (672 h) post-administration. Comparatively, ZA was only detectable up to 8 hours post-administration. Therefore, indirect detection with biomarkers can be advantageous to extend the time of detection up to 21 days (504 h) post-administration. OEA was increased and then further decreased in plasma levels extending the detection time to 14 days (336 h) post-administration. Used in combination HC (increase) and HC/C (increase) extended the detection time up to 21 days (504 h) post-administration. These biomarkers being specific to each administration with either complementary or indirect detection, would be beneficial for analysts to determine which type of BP was administered.

Using the semi-targeted results, Table 5.12 highlights the biomarkers showing a distinct pattern and the statistical testing to determine significance throughout the TA administration. Table 5.13 highlights the biomarkers that showed distinct activity past 8 hours post-administration where ZA was no longer detected and the statistical testing to determine significance.

Table 5-12: Semi-targeted biomarkers showing distinct pattern throughout a TA administration with statistical testing to determine significance.

Biomarker	Change observed	Time points post-administration	Fold Change	p-value	Statistically significant?
10-HDHA	Increase	21 days (504 h)	0.62	0.10	No
	Decrease	28 days (672 h)	-0.80	0.05	Requires further analysis
14-HDHA	Increase	21 days (504 h)	0.63	0.11	No
	Decrease	28 days (672 h)	-0.78	0.06	No
7-HDHA	Increase	21 days (504 h)	0.67	0.14	No

	Decrease	28 days (672 h)	-0.70	0.12	No
DHA	Increase	21 days (504 h)	0.36	0.04	No
	Decrease	28 days (672 h)	-0.44	0.02	No
EPA	Decrease	28 days (672 h)	-0.19	0.20	No

From the semi-targeted results, the biomarkers of 10-HDHA, 14-HDHA, 7-HDHA and DHA showed a similar pattern of being up regulated at 21 days (504 h) before becoming down-regulated at 28 days (672 h) post-administration. With EPA, it was only down-regulated at 28 days (672 h) post-administration compared to time 0. Whilst it is difficult at the present moment to determine if it is possible to detect these effects during race day samples as reference population wasn't completed. Additionally, compared to pre-administration samples, there is no statistical difference to the post-administration samples with the exception of 10-HDHA during a TA administration at 28 days (672 h) where the p-value was on 0.05 and DHA during a ZA administration at 4 days (96 h) post-administration where the FC was 1.98 and a p-value of 0.03.It would be wise to further validate these compounds to determine basal levels in both the general population and through control horses.

Table 5-13: Semi-targeted biomarkers showing distinct pattern after 8 hours post-administration during a ZA administration with statistical testing to determine significance.

Biomarker	**Change observed**	**Time points post-administration**	**Fold Change**	**p-value**	**Statistically significant?**
11-HEDE	Decrease	2 – 6 days (48 – 144 h)	-0.30 to -0.03	0.60 – 0.96	No
	Increase	28 days (672 h)	0.70	0.34	No
13-HODE	Increase	28 days (672 h)	0.76	0.26	No
14,15-DiHETE	Increase	28 days (672 h)	0.90	0.31	No
9-HODE	Increase	28 days (672 h)	0.50	0.36	No
20-HDHA	Increase	7 days (168 h)	0.67	0.23	No
	Decrease	21 – 28 days (504 – 672 h)	-0.28 to -0.23	0.46 – 0.54	No

DHA	Increase	3 days (72 h)	0.74	0.07	No
		4 days (96 h)	1.98	0.03	Yes
	Decrease	14 – 28 days (336 – 672 h)	-0.25 to -0.35	0.15 – 0.21	No
EPA	Increase	4 days (96 h)	1.89	0.01	Yes
		5 days (120 h)	0.76	0.02	No
		6 days (144 h)	0.61	0.03	No
		7 days (168 h)	0.55	0.03	No
		14 days (336 h)	0.01	0.71	No

Figure 5.28 summarises the biomarkers into a decision tree to differentiate between the two types of BP administrations. Ideally, an analyst would follow the path if certain biomarkers were observed to determine whether it was a non-nitrogenous or nitrogenous BP but there are a few biomarkers which are applicable to both. Therefore, not being able to differentiate but would aid the analyst to conclude that a BP was administered and recommend further testing to differentiate between the two types.

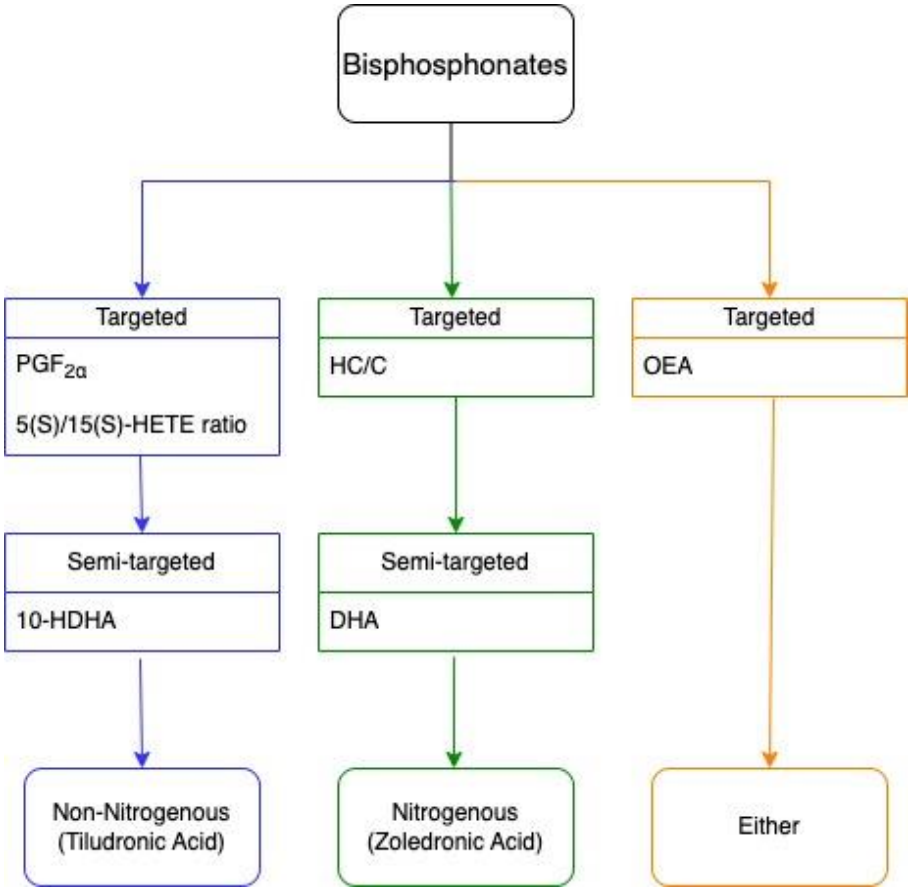

Figure 5.28: Decision tree based on analysis of biomarkers.

5.7 Bisphosphonate Conclusions

The use of BPs is of concern in the racing industry due to bone resorption properties. Whilst the elimination of TA in equine plasma has been extensively explored by other racing jurisdictions, the elimination of ZA in equine plasma in addition to the effects on endogenous compounds such as lipids and corticosteroids comparatively, have not been studied as extensively. Through the use of SPE and LC-MS/MS, ZA was detectable up to 8 hours post-administration which is consistent with the study conducted by Nieto *et al.* TA itself was detectable up to 28 days post-administration which is consistent with literature having seen TA detectable up to 3 years post-administration.

To extend the surveillance capability for BPs, lipid and corticosteroid biomarkers may be able to provide a complementary method of detection using LC-HRMS for a BP administration. It must be noted however, that the use of biomarkers is currently a novel technique in the

equine racing industry given that routine analysis relies on the detection of the prohibited substance[1]. Therefore, this approach would only be a complementary technique to the traditional detection of the prohibited substance and not as a full replacement. This study is considered preliminary for the indirect complementary detection of BPs. The validity of the proposed potential biomarkers needs to be further investigated by additional administration studies in different jurisdictions to explore alternative biomarkers to complement existing detection measures.

Compounds such as $PGF_{2\alpha}$, 18-HEPE, 15(S):5(S)-HETE ratio and 10-HDHA can potentially indicate a TA administration whilst HC/C ratio and DHA can potentially indicate a ZA administration. OEA can provide evidence that potentially a BP administration was given but aren't indicative of a nitrogenous or non-nitrogenous BP. The decision tree produced could be further expanded to hopefully incorporate other BPs to aid in the complementary detection of BPs using lipid and corticosteroid biomarkers.

Bisphosphonate References

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Chapter 6:
Research to Routine Translation

6.1 Rationale

The need for research in any field is necessary to improve methods to expand the scope of any laboratory's detecting capabilities. However, whilst research methods are heavily optimised for the detection of specific compounds, depending on the method, they can be difficult to incorporate into routine laboratory practices. These methods are likely to become stagnant methods once the research project finishes. Therefore, the need to extend research methods and effectively translate them into routine practice is necessary to ensure the laboratory's scope has been enhanced with the research completed.

With this rationale, 8 lipids and 3 corticosteroids that were affected by the previously discussed administrations (TACA, FLP and the bisphosphonates) were evaluated using routinely performed analytical procedures that were hypothesised to enable sufficient detection and quantification of these lipid biomarkers. The 100 µL sample volume utilised for research purposes was due to limited sample availability and harmonisation (as detailed in chapter 2.1.1). The use of the 2 mL sample was consistent with the routine laboratory operations determine if the lipid biomarkers could be extracted and analysed using the translated method. Ideally, these biomarkers could be further incorporated into routine monitoring in the future following an intensive population study.

6.2 Introduction

For the research to routine translation, the research extraction method was replaced in addition to the instrument used for analysis to the one that is currently used to monitor the acidic neutral fraction of equine plasma at ARFL. Table 6.1 highlights the differences between the two methods.

Table 6-1: Differences between the research to routine methods.

Method Step	Research method	Routine method
Sample amount	100 µL	2 mL
Protein precipitation solvent	0.1% formic acid in methanol	Trichloroacetic acid

Solid phase extraction cartridge	Phenomenex Strata-X reversed phase	UCT XTRACT mixed mode cartridge
Solid phase extraction elution solvent	0.1% formic acid in methanol	Ethyl acetate/hexane (3:2 v/v)
Reconstitution solvent	0.1% formic acid in methanol	0.1% formic acid in methanol and 0.1% formic acid in water (50:50)
Instrument	Shimadzu LC40 coupled to Shimadzu high resolution mass spectrometer (9030 QTOF)	Shimadzu Nexera LC-30AD coupled to Shimadzu triple quadrupole mass spectrometer (8060NX MS/MS)

As highlighted in table 6.1, there are numerous major alterations to the research method in comparison to the routine method. As methods use the acidic-neutral fraction of the equine plasma extraction, it is hypothesised that the extraction method will be able to isolate the lipid biomarkers using the routine method. If detectable, the lipid biomarkers will be able to expand the scope of target compounds currently being monitored in routine practices.

6.3 Materials and Methods

6.3.1 Chemicals and Reagents

Ammonium formate, LC grade ethyl acetate, formic acid (FA), hexane and methanol (MeOH) were purchased from Merck (Darmstadt, Germany). Formic acid (FA) and methanol (MeOH) of MS grade were purchased from ThermoFisher (Waltham, Massachusetts, USA). Trichloroacetic acid (TCA) was purchased from Sigma Aldrich (Castle Hill, NSW, Australia). Water used was ultrapure grade (18.2 MΩ cm) obtained from a ThermoFisher Barnstead Smart2Pure system (Langenselbold, Hungary).

6.3.2 Routine Race Day – Blood Samples

All blood samples (pre-race) were collected in BD Lithium Heparin Vacutainer® (Mississauga, ON, Canada) tubes (Cat # 367880) by veterinarians and swab officials employed by Racing NSW. Samples were transported chilled to the Australian Racing Forensic Laboratory (ARFL)

where, on arrival and after checking integrity (e.g., seals) of samples, they were stored at 4 °C until extraction for analysis.

6.3.3 Sample Preparation

Sample blood tubes were centrifuged at 3000 rotations per minute (rpm) for 10 minutes using a Beckman Coulter Allegra X-12 centrifuge. Following this, 2 mL of equine plasma from each sample was transferred to 13 mL borosilicate glass tube. A mix of internal standards including Prostaglandin $F_{2\alpha}$ – D_4, 15(S)-HETE-D_8, 5(S)-HETE-D_8 and 14,15-DiHET-D_{11} at 2000 ng/mL, OEA-D_4 at 4 ng/mL and Hydrocortisone-D_4 at 37.5 ng/mL was added to each sample before a vortex and addition of 200 µL of TCA (10% v/v) to generate a protein precipitate. Water (3 mL) was added, and pH adjusted to between 3 and 3.5 using dilute HCl (10% v/v or 3% v/v) and verified using a pH electrode (Cole-Parmer, Vernon Hills, IL, USA) coupled to a pH meter (Radiometer analytical (MeterLab), Lyon, France) . Samples were then centrifuged at 3000 rpm for 10 minutes to separate the protein pellet and obtain the supernatant for SPE.

SPE was performed using a UCT positive pressure manifold with UCT Xtrackt mixed mode (200 mg, 3 mL) cartridges (Lewiston, PA, USA). The cartridges were pre-conditioned with 3 mL of MeOH and water consecutively. Sample was then loaded onto the cartridge and further washed with 3 mL of 0.1 M acetic acid solution and pushed dry with nitrogen. The acidic-neutral fraction was then eluted using a solution of ethyl acetate and hexane (3:2 v/v, 3 mL).

The eluate was dried under a gentle stream of nitrogen using a heating block set to 60 °C. The dried residue was reconstituted in 50 µL of 0.1% FA in MeOH and 50 µL of 0.1% FA in H_2O. Samples were transferred to an autosampler vial and stored at 4 °C until LC-MS/MS analysis.

6.3.4 Liquid Chromatography Tandem Mass Spectrometry (Triple Quadrupole)-Instrument Parameters

LC-MS/MS analysis was performed using a Shimadzu Nexera X2 LC-30AD UHPLC coupled to an 8060-NX triple quadrupole mass spectrometer. The LC system was equipped with a Phenomenex Luna C18 (100 mm x 2 mm, 3 µm) column using a binary gradient. Mobile phase A consisted of aqueous ammonium formate (5 mM, pH 3.0) and mobile phase B consisted of

0.1% FA in MeOH. The run time was 21.50 minutes with the following gradient: 0-1 minutes A-B (95:5 % v/v), 1-2 minutes A-B (99:1 % v/v), 2-15 minutes A-B (5:95 % v/v), 15-19.20 minutes (5:95 % v/v), 19.20-19.25 minutes (95:5 v/v) and at 19.25-21 minutes, the mobile phase was brought back to initial conditions. The flow rate was constant at 0.4 mL/min with an injection volume of 2 µL.

Shimadzu's LabSolutions (version: 5.113) was used for data acquisition using a National Association of Testing Authorities (NATA) accredited method at the ARFL for the analysis of the acidic-neutral compounds in equine plasma. Data was acquired in ESI positive and negative mode with the MS conditions stated in appendices A22.

6.3.5 Method Validation

The LC-MS/MS method in 6.3.4 was validated for the quantification of OEA and AEA, following on from the method validation for hydrocortisone and cortisone presented previously[93]. Due to the endogenous content of both OEA and AEA, equine plasma used for method validation underwent LLE using DCM:EtOH (90:10 v/v) to reduce the baselines and improve low-level quantification. For the produced surrogate matrix, 91% of OEA and 65% of AEA were removed (Table 2.14). The parameters assessed were discussed in chapter 2.2.7 method validation preparation. linearity, sensitivity, accuracy, precision, recovery, matrix effects and stability as outlined by Peters *et al*[99].

6.3.5.1 Linearity

Linearity was assessed as described in chapter 2.2.7.1 with spikes in the DCM:EtOH surrogate matrix equine blank plasm due to the endogenous levels present. Concentrations of 0, 1, 2, 5, 10 and 50 were used for OEA and AEA as spikes in the plasma matrix. Working solutions of 100 ng/mL were made up from the 2000 ng/mL working solution. The spike amounts were determined using equation 2.1 (in chapter 2.2.7.1). The amounts used for each concentration can be visualised in appendices table A23. Plasma spikes were replicated four times to account for any variabilities which may be associated with either the sample preparation or instrumental analysis. Regression analysis was also completed in accordance with chapter 2.2.7.1.

6.3.5.2 Sensitivity

The method sensitivity was assessed by estimating the LOD and LOQ as set out in chapter 2.2.7.2 for OEA and AEA. Using the signal-to-noise ratio (*S/N)*, the comparison could then be made for a clear comparison for a *S/N* of 3 for the LOD and a *S/N* of 10 for the LOQ[100]. Concentrations of 0.05, 0.04, 0.03, 0.025, 0.02 and 0.01 ng/mL were chosen with Table A24 in appendices visualising the total amounts required for a 1 ng/mL working solution with this working solution being made from a 2000 ng/mL stock solution.

6.3.5.3 Accuracy

Accuracy was assessed in a similar manner to those as set out in chapter 2.2.7.3 and using equation 2.3. Concentrations were estimated using plasma spikes at 5 ng/mL for OEA and 1 ng/mL for AEA.

6.3.5.4 Precision

Precision was estimated in a similar manner to chapter 2.2.7.4 using equation 2.4. Concentrations used for precision were the same as accuracy (5 ng/mL for OEA and 1 ng/mL for AEA).

6.3.5.5 Recovery

Recovery was assessed in a similar manner to chapter 2.2.7.5 using equation 2.5. Concentrations to assess recovery was the same as accuracy and precision (5 ng/mL for OEA and 1 ng/mL for AEA).

6.3.5.6 Matrix Effects

Matrix effects (ME) assessed using the conditions as described in chapter 2.2.7.6 using equation 2.6. Concentrations used to assess ME were the same as accuracy, precision, and recovery (5 ng/mL for OEA and 1 ng/mL for AEA).

6.3.5.7 Stability

Stability was assessed over a 3-month period with samples analysed at 1-month, 2- months and 3-months at the same concentrations as set out previously (5 ng/mL for OEA and 1 ng/mL for AEA) stored at either 4 °C or – 20 °C in duplicate. At the allocated time point, the samples were thawed to room temperature and prepared using the method as outlined in chapter 6.3.3. Concentrations were calculated utilising the LabSolutions Insight software. The average concentration for each temperature was taken over their associated time period using the average function in *Excel*.

6.3.6 Data Processing Parameters

All data processed on the LC-MS/MS 8060NX was analysed using LabSolutions Insight (Version 3.8 SP3) with further data processing completed on Excel (version 16.71), MATLAB (Version R2021A) or MetaboAnalyst (version 5.0).

6.3.6.1 Raw Data Processing

The method for raw data includes each compound, the parent and transition ions and the relative internal standard the compound was being quantified with. The concentrations from the instrument were calculated using linear regression with the calibration curve being the area ratio of the target compound to the internal standard response for the respective concentration. Table 6.2 shows the criteria the LabSolutions Insight software had for the quantification of the compounds.

Table 6-2: Parameters for LabSolutions Insight for acquisition data for LC-MS/MS 8060

Condition	Optimised Condition
Peak Integration Conditions	
Smoothing Method	Standard
Smoothing Width	2 seconds
Identification Conditions	

Identification Method	Absolute
Peak Selection	Closest Peak
Window for Target Peak	2.00 %
Window for Reference Peak	5.00 %
Processing Time	± 0.5 min
Quantitative Conditions	
Method	Internal Standard
Calculated by	Area
Number of calibration levels	8
Type	Linear
Zero	Not Forced
Units	ng/mL

6.3.6.2 Reference population data analysis and statistics

Data analysis was completed using *Microsoft Excel for Mac* (version 16.76) using basic statistical functions. Functions include the mean, standard deviation, frequency, kurtosis, skewness, and quartile analysis. Boxplots and student's T-Tests for gender comparisons and normality population plots was completed using MATLAB (version R2023b) utilising the functions of the additional Statistics and Machine Learning Toolbox (version 23.2). Results from the student's T-Test indicating p-values greater than 0.05 showed statistical difference between the genders whilst p-values less than 0.05 indicated no statistical difference.

6.4 Results and Discussion

6.4.1 Liquid Chromatography Conditions

A routine method was utilised for the research to routine translation. This was to determine if the addition of the chosen biomarkers could be detected without affecting other analytes

already being monitored. Chromatographic separation of target compounds was verified using the routine LC conditions. Retention times for each compound were checked with the highest calibrator for each sequence to ensure compliance with Association of Official Racing Chemists (AORC) criteria[101]. Each retention time was expected to fall within ± 0.2 minutes of the corresponding reference material [101] with table 6.3 highlighting the retention time for the compounds being translated from the research method to routine method. Relative retention time was calculated using equation 2.13 from chapter 2.3.1.

$$Relative\ Retention\ Time = \frac{Retention\ Time\ of\ Compound}{Retention\ Time\ of\ Internal\ Standard\ Used}$$ (Equation: 2.13)

Table 6-3: Retention time and relative retention time for translated biomarkers under the optimised LC conditions.

Compounds	Retention Time (minutes)	Internal Standard	Relative Retention
18-Hydroxycortisol	8.98	D_4-Hydrocortisone	0.88
Cortisone	9.82	D_4-Hydrocortisone	0.96
Hydrocortisone	10.1	D_4-Hydrocortisone	0.99
Prostaglandin $F_{2\alpha}$	12.1	D_4-Prostaglandin $F_{2\alpha}$	1.01
9-HOTrE	14.1	14,15-DiHET-D_{11}	1.01
18-HEPE	14.2	14,15-DiHET-D_{11}	1.02
15-HETE	14.6	15-HETE-D_8	1
12-HETE	14.8	5-HETE-D_8	0.99
5-HETE	14.9	5-HETE-D_8	1
AEA	15.6	D_4-OEA	0.98
OEA	16	D_4-OEA	1

6.4.2 Optimised Mass Spectrometry Conditions

Using the routine MS conditions, MRM transitions were optimised and selected to ensure specificity based on the mass to charge ratio (*m/z*) of the target compound. The optimised MS conditions are provided in table 6.4.

Table 6-4: Optimised MS conditions for translated biomarkers (*indicates transition used to quantify).

Compounds	ESI (+/-)	Precursor ion (*m/z*)	Product ion (*m/z*)	Collision Energy (CE)
OEA	Positive	326.15	62.15*	-17
			309.30	-16
			121.20	-23
			97.05	-25
			69.05	-31
18-Hydroxycortisol	Positive	379.05	267.20*	-21
			285.25	-17
			121.20	-30
Hydrocortisone	Positive	363.00	121.15*	-22
			159.00	-17
			309.20	-17
			267.25	-19
			327.25	-16
Cortisone	Positive	361.15	162.90*	-24
			121.15	-31
			135.05	-26
AEA	Positive	348.20	62.20*	-17
			287.25	-14
			203.20	-17
			245.25	-17
Prostaglandin $F_{2\alpha}$	Negative	353.15	193.25	27
			309.20*	20
			291.10	21
			247.15	23

15-HETE	Negative	319.15	219.30*	13
			257.35	15
			147.00	23
			175.30	15
5-HETE	Negative	319.85	115.10	14
			257.25	14
			203.20	18
			301.05*	11
12-HETE	Negative	319.20	179.10*	14
			301.00	15
			257.20	14
			208.10	14
9-HOTrE	Negative	293.45	171.10*	16
			159.10	16
			115.10	25
			283.35	23
18-HEPE	Negative	316.95	113.10	15
			181.10	10
			248.90	9.0
			215.15*	16
Prostaglandin $F_{2\alpha}$-D_4	Negative	357.25	313.35*	20
15-HETE-D_8	Negative	327.20	226.15*	13
5-HETE-D_8	Negative	327.20	264.50*	15
14,15-DiHET-D_{11}	Negative	348.20	207.15*	20
OEA-D_4	Positive	330.20	66.05*	-17
Hydrocortisone-D_4	Positive	367.15	121.00*	-24

6.4.3 Method Validation of Biomarkers

Method validation for the routine method was completed for OEA and AEA. The method validation for HC and C were completed in a previous study[93].

6.4.3.1 Linearity

A calibration curve was used to establish the linearity of the target compounds. The R^2 value is indicative of how linear the curve is with values as close to 0.999 being ideal. Table 6.5 indicates the R^2 value using data obtained from 4th to 26th of April 2023. Figure 6.1 shows the calibration curve for OEA over the duration of 4 weeks and Figure 6.2 shows the calibration curve for AEA spanning the same timing as OEA. The R^2 value is calculated to also be 0.9999 also indicating a linear curve.

Table 6-5: R^2 values for OEA and AEA.

Compounds	**R^2 value (Averaged over 4 batches)**	**R^2 value (From instrument on 4th of April 2023)**
OEA	1.00	0.9993
AEA	1.00	0.9999

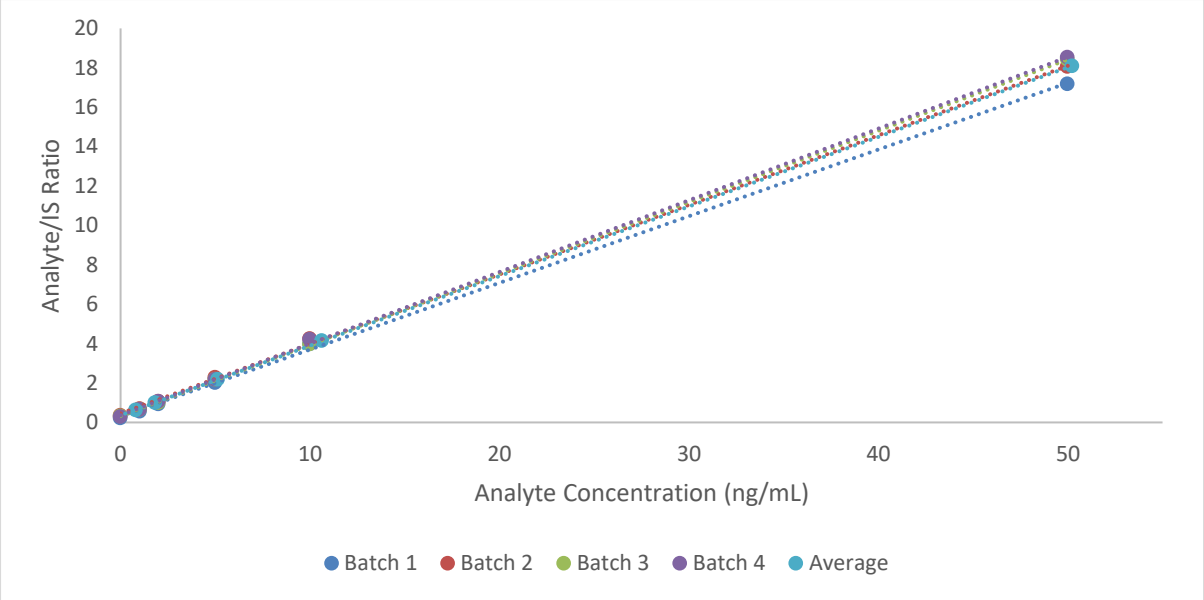

Figure 6.1: Linearity of OEA in equine plasma (2 mL) over 4 separate batches. Batch 1 equation = y = 0.3384x + 0.3161 (R^2 = 0.9995), Batch 2 equation: y = 0.3529x + 0.4537 (R^2 = 0.9995), Batch 3 equation: y = 0.362x + 0.3243 (R^2 = 1), Batch 4 equation: y = 0.3639x + 0.3598 (R^2 = 0.9998), Average equation: 0.3532x + 0.3631 (R^2 = 1)

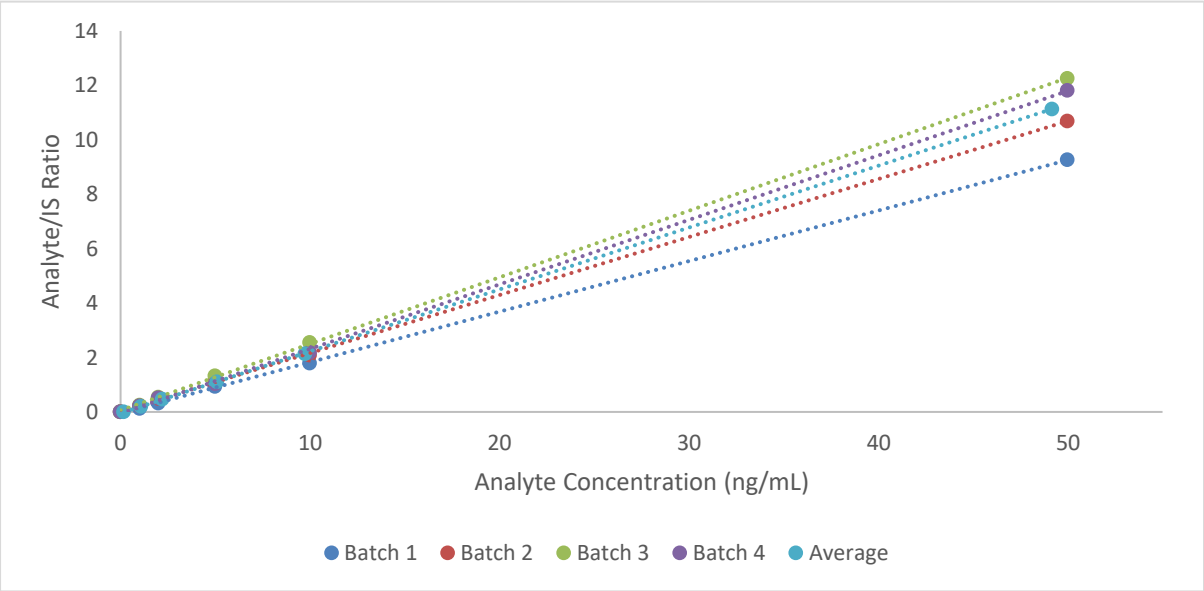

Figure 6.2: Linearity of AEA in equine plasma (2 mL) over 4 separate batches. Batch 1 equation: y = 0.1858x – 0.0305 (R^2 = 0.9999), Batch 2 equation: y = 0.2131x + 0.0317 (R^2 = 0.9999), Batch 3 equation: y = 0.2445x + 0.055 (R^2 = 0.9999), Batch 4 equation: y = 0.2274x – 0.048 (R^2 = 0.9996), Average equation: y = 0.2274x – 0.048 (R^2 = 1).

Further assessment of the linearity of the calibration curve was completed using y-residuals. Whilst accuracy is important between analysis batches, the residual are calculated to determine if there is an underlying bias in the calibration[137] . Figure 6.3 demonstrates the residuals for OEA and AEA. The residual plots were completed using the same 4 calibration used for linearity. Looking at the y-residuals for both OEA and AEA, there is no presence of bias amongst the spiked samples due to the variability in the graphs.

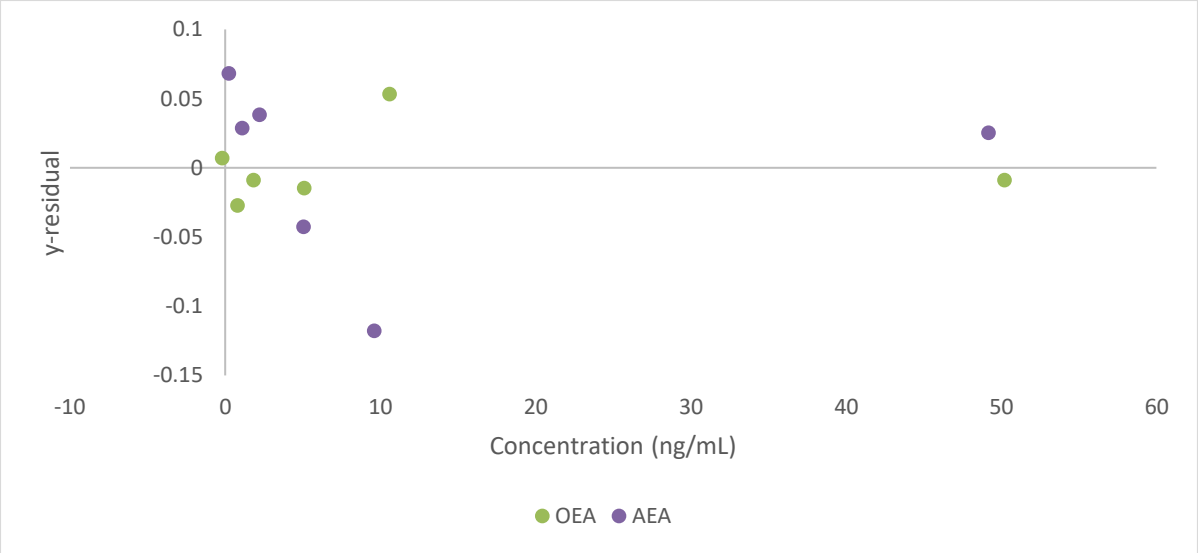

Figure 6.3: Average y-residual for OEA and AEA in equine plasma over 4 separate calibrations.

6.4.3.2 Sensitivity

The visual method was used instead of linear regression as linear regression produced inappropriate detection limits. For chromatograms to determine the decision limits can be seen in figures A56 and A57 in the appendices. The LOD of both OEA and AEA was estimated to be less than 0.01 ng/mL due to the inability to spike accurately below 0.01 ng/mL with the endogenous component still slightly present in the surrogate matrix plasma. The LOQ was estimated at 0.02 ng/mL for both OEA and AEA.

6.4.3.3 Accuracy, Precision, Recovery and Matrix Effects

Accuracy was performed using replicates of 7 with the accuracy being determine using percentage relative error (% RE). Table 6.6 shows the results for accuracy with the deemed concentrations needing to be equal to or less than 15% for the conclusion that samples were accurate. With this condition, however, only OEA is deemed accurate for this method. AEA fell outside of this limit which could be attributed to the lower concentration used (1 ng/mL) compared to OEA or the extraction method itself isn't optimal for AEA.

Precision was assessed using the relative standard deviation (% RSD) using the same 7 replicate set as used for accuracy. Results for precision can be seen in table 6.6 with samples being deemed precise if the % RSD is equal to or less than 15%. Both OEA and AEA were deemed precise using this method.

Both OEA and AEA were assessed using replicates of 7 to determine the recovery with the area ratios used for comparison. Table 6.6 visualises the recovery for both OEA and AEA at the selected concentrations. A good recovery is expected to be higher than 50% however, for both OEA and AEA, these fell short of this expectation. This could be due to relatively poor extraction for these two compounds using the routine SPE method designed for a wide range of compounds. Haemolysed plasma can contribute to such low SPE recoveries. There is also the possibility of inconsistent spiking between the replicates for lower recovery.

ME was assessed using the same replicates of 7 used for post-extraction recovery in addition to a set of 7 replicate neat standard spikes. Both OEA and AEA showed consistent ion suppression due to the ME being lower than 100% for both. The use of the 7 replicates ensured the reliability of this result.

Table 6-6: Results from accuracy, precision, recovery, and matrix effects

	Concentration (ng/mL)	**Accuracy (% RE)**	**Precision (% RSD)**	**Recovery (%)**	**Matrix Effects (%)**
OEA	5	7.7	4.9	25	96
AEA	1	25	5.7	46	79

6.4.3.4 Stability

Stability is an essential component of the method validation process as many samples are stored in certain environments before they are needed for processing or analysis. The ARFL has a quick turn over period for samples from receipt to reported result, they are stored for no more than one month. However, for method validation purposes, it is important to assess the stability of compounds over a longer period of time The stability study was completed in the same concentrations as previous method validation components for a 3-month period at the temperatures of 4 °C or – 20 °C. Figures 6.4 and 6.5 provides the results of the stability study conducted for OEA and AEA. OEA was deemed stable for a 2-month period, but OEA did indicate the presence of degradation at the 3-month period being lower than half of the original concentration therefore, being deemed stable for a 2-month period. AEA, however, was deemed stable for a 1-month period. The later months of AEA could not be deemed stable with the 3-month stability having a higher percentage compared to the 2-month stability. This is likely due to the low recovery of this method as seen in table 6.6 with a 46% recovery for AEA affecting the concentrations obtained for AEA between 2 to 3 months.

Comparing the stability study conducted for the 100 µL method and the routine method. AEA and OEA had a consistent stability of 1 month and 2 months respectively. Comparatively using the 100 µL method, both AEA and OEA were stable for 4 weeks. As the stability for the 100 µL method was only completed to 4 weeks due to timing of the method validation, it is unknown whether OEA would be stable for a similar time period. As AEA produced a similar stability however, it can be hypothesised that OEA would follow a similar pattern if this stability study was completed in the same time frame.

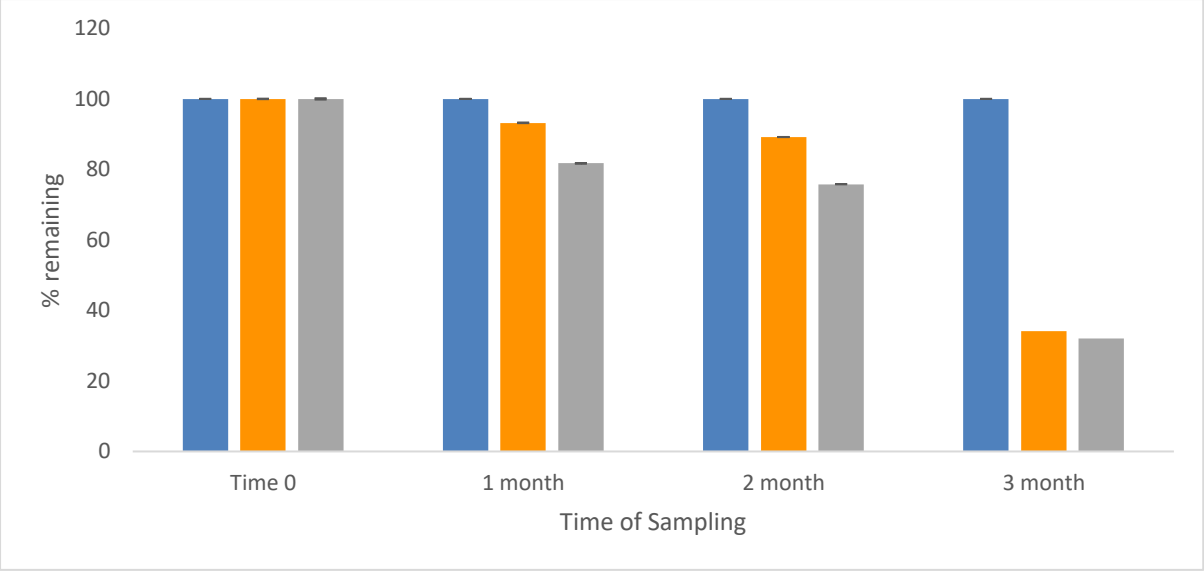

Figure 6.4: Stability results for OEA at 4 °C and -20 °C (Blue indicates Time 0, orange indicates 4 °C and grey indicates -20 °C).

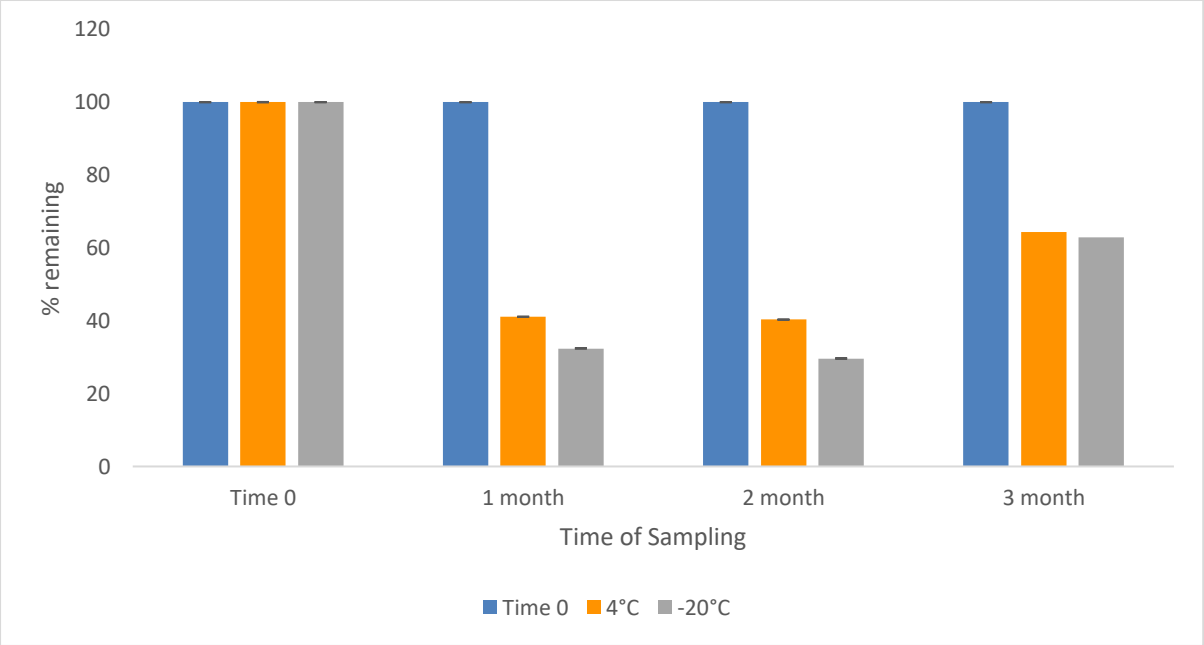

Figure 6.5: Stability results for AEA at 4 °C and -20 °C (Blue indicates Time 0, orange indicates 4 °C and grey indicates -20 °C).

6.4.4 Reference Population for OEA/AEA

Reference population samples were compiled using race day samples collected prior to the race commencing. Table 6.7 lists the number of samples for this chapter of the study with a

total of 268 samples analysed. This set of samples was collected separately to the samples in chapter 2.4 and without a freeze thaw cycle.

Table 6-7: Number of samples analysed through the routine method for the reference population.

Grouping	Gender	Sample Amount
Pre-Race race day	Female	105
	Gelding	98
	Male	65

Using the routine method revealed that majority of the compounds (i.e. 18-hydroxycortisol, $PGF_{2\alpha}$, 15(S)-HETE, 5(S)-HETE, 12(S)-HETE, 9-HOTrE and 18-HEPE) were either below LOD or were not quantifiable during the study. Therefore, these 6 compounds were not further analysed. The only two compounds from herein to be discussed for the research to routine translation is OEA and AEA to also coincide with the 100 µL reference population data. From the 268 samples analysed, 93% of OEA and 70% of AEA was detectable with concentrations not exceeding 5.47 ng/mL for OEA and 0.67 ng/mL for AEA. Concentrations of AEA were deemed as estimations due to the method not being successfully validated for accuracy.

From each set of data, concentrations that were below the LOQ of 0.02 ng/mL for both OEA and AEA were removed. After the removal of concentrations below the LOQ, OEA had a total of 249 samples whilst AEA had a total of 187 samples analysed using the routine method. From the reference population data shown in Table 6.8, this outlines the average concentration, standard deviation, and median concentration for the two markers.

Table 6-8: Results for OEA and AEA (estimated concentrations for AEA) from reference population.

Compounds	Total number of samples	Gender	Number of Samples	Average Concentration (ng/mL)	Standard Deviation	Median Concentration (ng/mL)
OEA	249	Female	96	1.5	1.2	1.2

		Gelding	91	1.5	1.3	1.3
		Male	62	1.5	0.7	1.3
AEA	187	Female	59	0.3	0.1	0.3
		Gelding	77	0.3	0.3	0.3
		Male	51	0.3	0.07	0.3

Box plots were generated in MATLAB to determine if there were any significant differences between the three genders. It was expected that the student's t-test would show similar results and indicate no significant difference between the three sets of data. From the student's T-Test, OEA had values of $p > 0.05$ indicating no significant difference. Comparatively, for AEA, the gelding to male comparison yielded a value of $p > 0.05$ showing no significant difference however, for the female to gelding and female to male comparison yielded $p < 0.05$. This can be due to the very low estimated concentrations of AEA, with any deviation, this could lead to a p value indicating significant difference. Therefore, the box and whisker plots for OEA and AEA were generated. Figures 6.6 to 6.7 highlights the boxplots for OEA and AEA respectively.

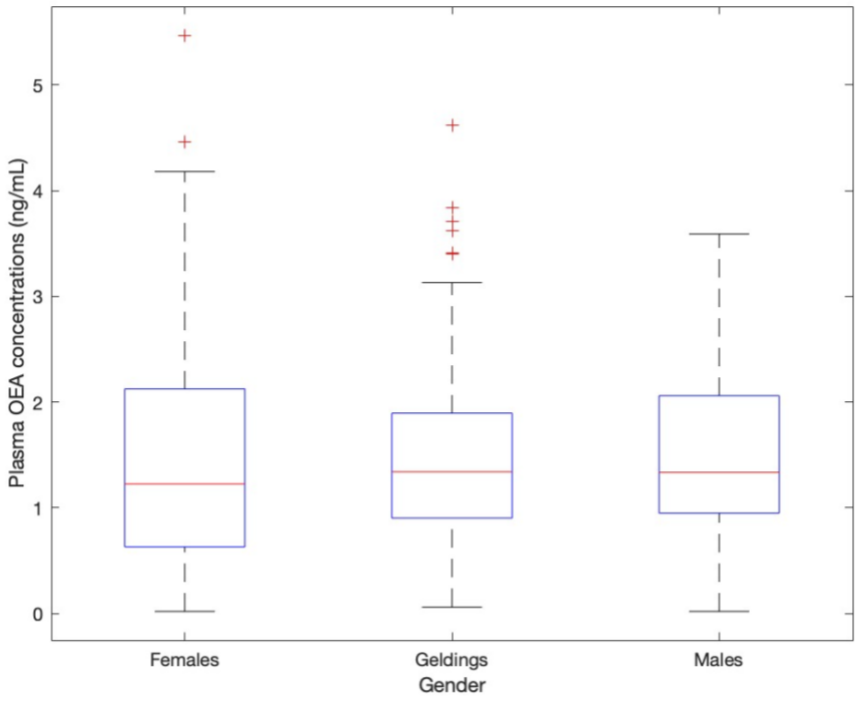

Figure 6.6: Box plot for the gender comparison for OEA (n=249)

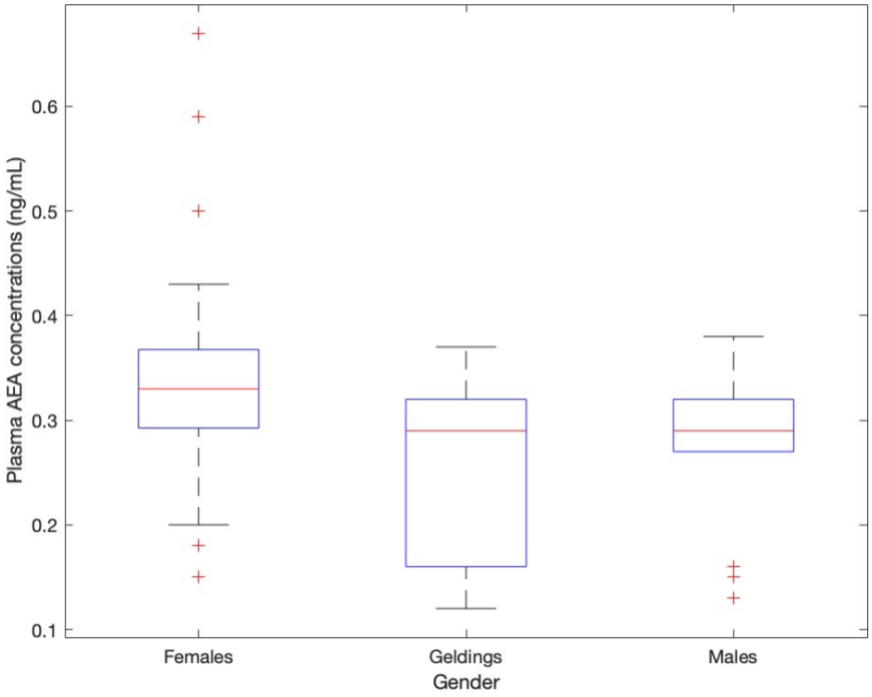

Figure 6.7: Box plot for the gender comparison for AEA using estimated concentrations (n=187).

From the boxplots in Figures 6.6 to 6.7, there does not appear to be a significant difference between the three genders. It should be noted that due to the low values for AEA, as the variation is still low, it can be concluded that the significant difference is not there based off of the box and whisker plots. This conclusion has allowed for combined sets of data to be used for any proposed thresholds.

6.4.4.1 OEA as a biomarker

OEA concentrations as seen in Figure 6.8 ranged from 0.06 ng/mL up to 5.47 ng/mL. Samples below the 0.02 ng/mL LOQ were removed from the data set as these were deemed undetectable. There are various possibilities for this with individual horses having their own individual levels of OEA. As OEA is as at the top of the AA cascade, the presence of OEA is expected but it does require metabolism from AEA as its analogue, it is likely the lower concentration is due to the extra step required to metabolise. As OEA is also an endogenous cannabinoid receptor, as these samples were taken pre-race, it is hypothesised that the horses are subject to a level of stress which would affect their feelings of calmness.

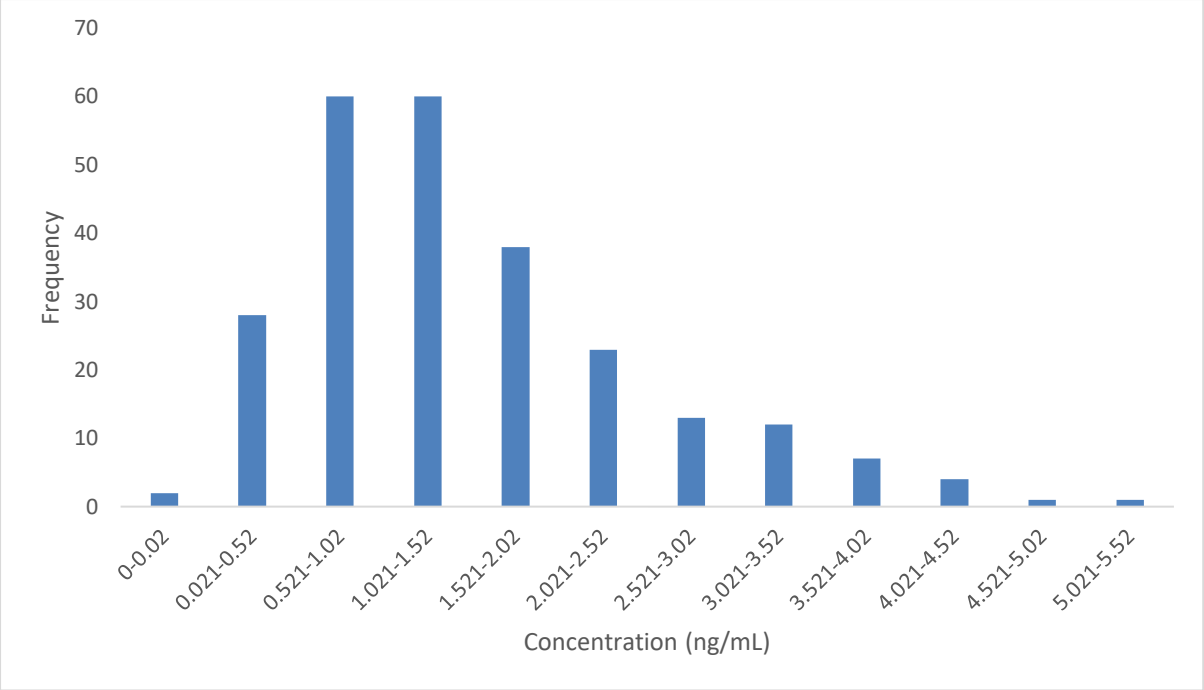

Figure 6.8: Frequency distribution for the concentration of OEA in equine plasma for all gender (n=249).

The overall aim for the screening of lipids using the routine method was to determine if the lipids could be added to monitor or complement existing methods. This would include the possibility of proposing upper and lower thresholds for the introduction of a new biomarker to monitor. With the overall aim to potentially suggest an international OEA threshold in plasma, this can occur with a parametric distribution, therefore a log normal transformation of the concentration is required. Removing the outliers from the data set by removing those below the LOQ of 0.02 ng/mL, Figure 6.9 shows a parametric distribution for OEA for all genders.

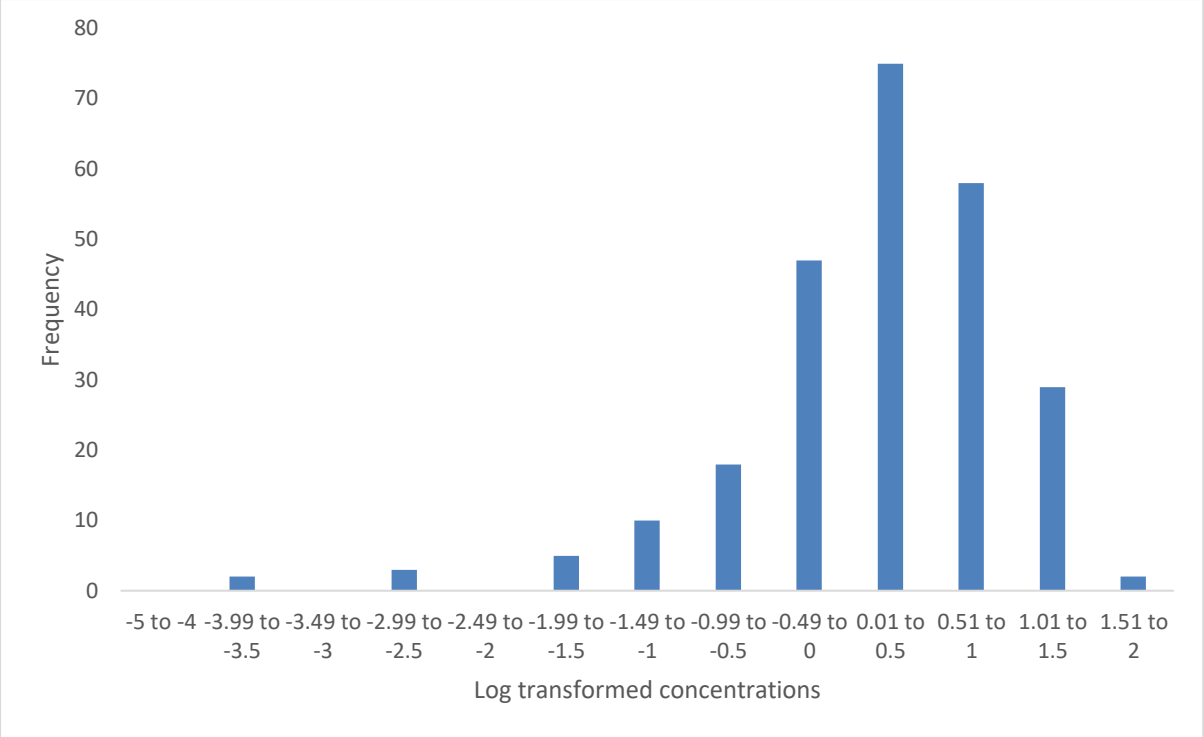

Figure 6.9: Frequency distribution for the log transformed concentration of OEA in equine plasma for all genders (n=249).

In Figure 6.9, there appears to be a normal distribution however, there is a left end tailing on the lower low normal transformed figures. The normality of this set of data was tested using kurtosis and skewness resulting in 3.38 indicating a data set that is heavily tailed. The skewness test resulted in a figure of -0.98 indicating a skewness to the left as seen in Figure 6.9.

A normal probability plot in MATLAB was also conducted to test for the normality of the data. This test plots the log transformed values against the initial concentration to determine the range of data that is parametric. Figure 6.10 shows the normality probability plot for OEA. According to this figure, the parametric range for OEA spans from log -0.63488 to 1.2238 which equals to 0.53 ng/mL to 3.4 ng/mL. Therefore, from the total count of 249 samples analysed, 89.7% is parametric.

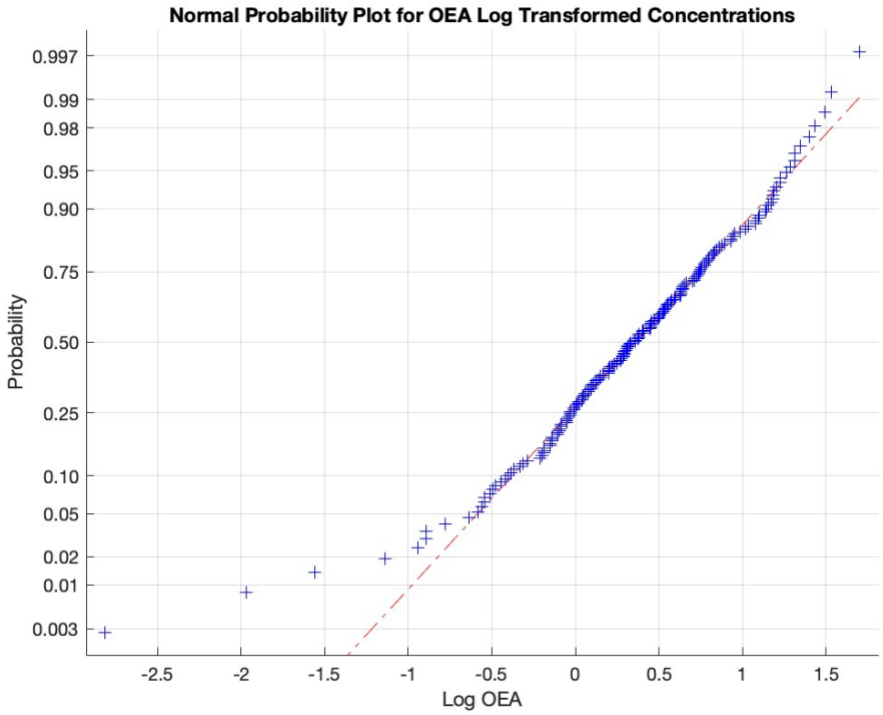

Figure 6.10: Normality probability plot for OEA log transformed concentrations.

Using the parametric range from the log transformed values, this dataset can be used to propose a threshold for OEA concentrations using Equation 2.7 in chapter 2.2.9. Using the mean, standard deviation and formula, Table 6.10 indicates the results from the equation.

Table 6-9: Results from the threshold equation for OEA in equine plasma using the parametric range.

Transformations	**Result**
Average log transformed concentration	0.34
Standard deviation of log transformed concentration	0.46
Threshold = Mean + (Standard Deviation x 3.72)	2.1
Threshold = Mean - (Standard Deviation x 3.72)	1.4
Upper threshold (ng/mL)	8.0
Lower threshold (ng/mL)	3.9

From the analysis of OEA concentrations in equine plasma using 184 samples, Equation 2.7 determines the thresholds of 8.0 ng/mL as the proposed upper threshold and 3.9 ng/mL as the proposed lower threshold. These figures have been proposed with the large assumption that there is no presence of any therapeutics that affect OEA, have had their spectra analysed properly with the correct integration and no other outstanding features having influenced the data. In a similar manner to the 100 µL method, the thresholds of 8.0 and 3.9 ng/mL is quite high being half of the upper threshold of 8.0 ng/mL. This could be due to the low recovery seen with the routine method being only 25% during method validation. From this, a non-parametric workflow to determine the population reference limits with table 6.11 highlighting the upper and lower thresholds. As this is in reference to non-parametric statistics, the original data set of 249 samples was used for the threshold calculations.

Table 6-10: Results from threshold calculations for OEA in equine plasma using non-parametric statistics (n=249).

Calculations	Population Reference Limit (ng/mL)
Upper threshold	5.5
Lower threshold	0.02

From the non-parametric statistics, the upper and lower thresholds were calculated to be 5.5 ng/mL and 0.02 ng/mL (the LOQ). These concentrations appear to be more appropriate given the concentration range of OEA with consistency to the 100 µL method. The 100 µL method generated upper and lower thresholds of 5.6 ng/mL and 0.21 ng/mL (the LOQ) which is very similar to the routine method. Further work with a larger number of samples from interstate and international laboratories will need to be completed to determine if concentrations are similar.

6.4.4.2 AEA as a biomarker

As quantification of AEA could not be fully validated, all concentrations of AEA are considered estimations. Estimated AEA concentrations in comparison to OEA remained consistent throughout the entire reference population ranging from the minimum estimated concentration of 0.12 ng/mL to the maximum at 0.67 ng/mL. From the reference population

data set utilised in a similar manner to OEA, the same process was completed to determine whether a parametric distribution was evident. Figure 6.11 visualises the distribution for AEA log transformed concentrations for all three genders.

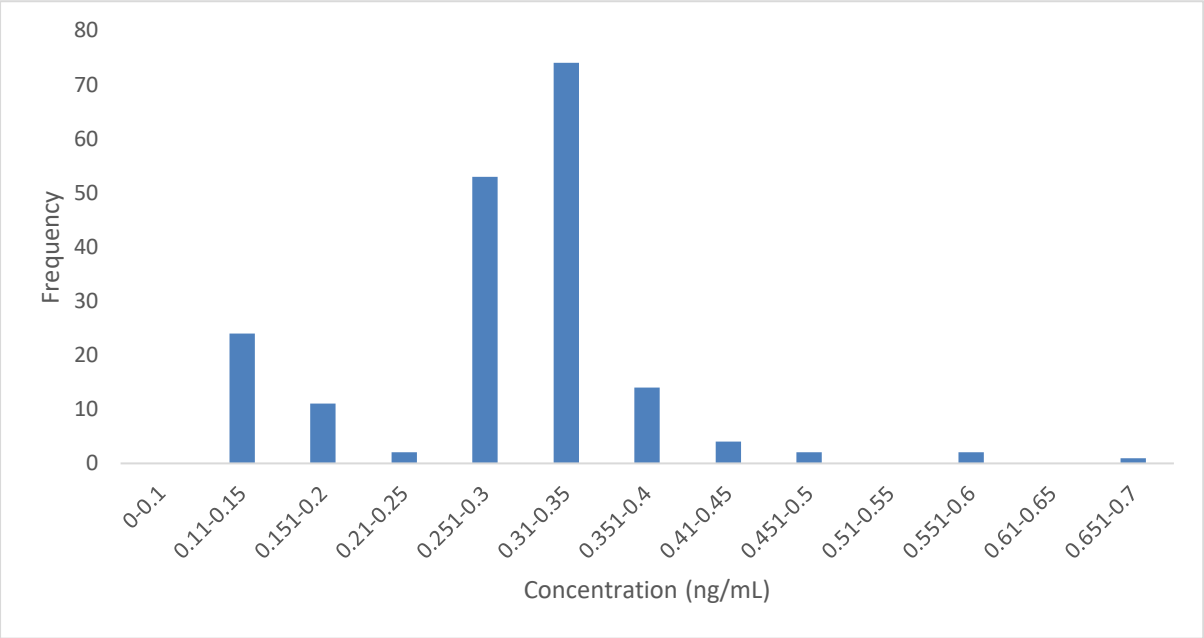

Figure 6.11: Frequency distribution for the log transformed estimated concentration of AEA in equine plasma for all genders (n=187).

Similar to OEA concentrations, AEA appears to also follow a parametric distribution. Figure 6.12 shows the log normal transformed estimated concentrations of AEA.

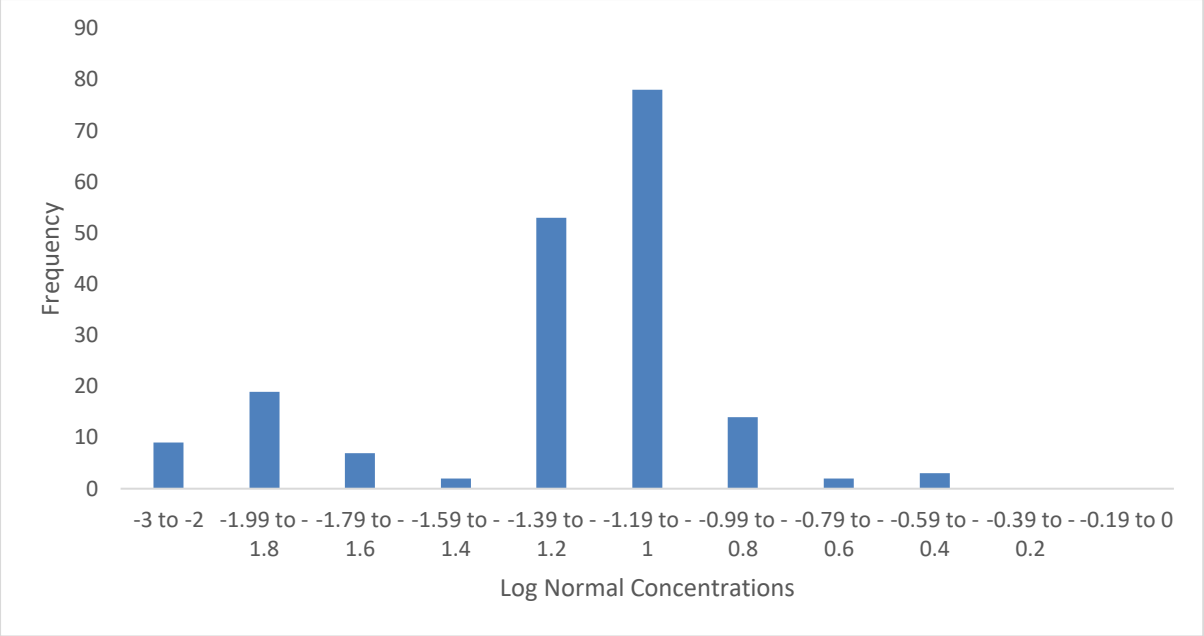

Figure 6.12: Frequency distribution for the log transformed estimated concentration of AEA in equine plasma for all genders (n=187).

Using the kurtosis and skewness test, these resulted in a kurtosis of 4.77 and skewness of -1.61. Similarly, using the normal probability plot from MATLAB, Figure 6.13 highlights the parametric range for AEA. According to this figure, the parametric range for AEA spans from log -1.3093 to -0.99425 which estimates to 0.43 ng/mL to 4.5 ng/mL. Therefore, from the total count of 187 samples analysed, 73% is parametric.

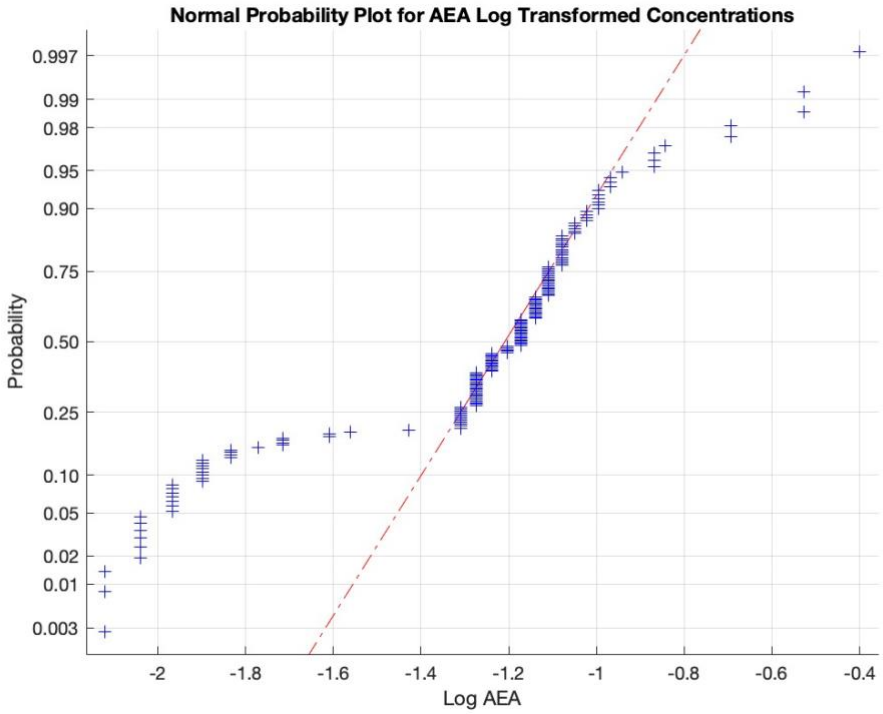

Figure 6.13: Normal probability plot for AEA log transformed estimated concentrations.

Using the parametric range from the log transformed values, this dataset can be used to propose a threshold for AEA concentrations using Equation 2.7 in chapter 2.2.8.2. Using the mean, standard deviation and formula, Table 6.12 indicates the results from the equation.

Table 6-11: Results from the threshold equation for AEA in equine plasma using the parametric range.

Transformations	**Result**
Average log transformed estimated concentration	-1.17
Standard deviation of log transformed concentration	0.09
Threshold = Mean + (Standard Deviation x 3.72)	1.5
Threshold = Mean - (Standard Deviation x 3.72)	-0.83
Estimated upper threshold (ng/mL)	4.5
Estimated lower threshold (ng/mL)	0.43

From the analysis of estimated AEA concentrations in equine plasma using 187 samples, the equation 2.7 determines the thresholds of 4.5 ng/mL as the estimated upper threshold and 0.43 ng/mL as the estimated lower threshold. Given the consistency of the estimated AEA concentrations, any deviation from these thresholds would be highly suggestive of an exogenous drug being administered. In comparison to the 100 µL method, the estimated thresholds are not as consistent. The 100 µL method estimated a threshold of 0.57 ng/mL as the upper threshold and 0.2 ng/mL as the lower whilst the routine method estimated 4.5 ng/mL and 0.43 ng/mL as the upper and lower threshold respectively. The main difference is the 100 µL set was not deemed parametric whilst the routine method was 73% parametric. This is likely due to the increase in matrix providing a more accurate estimation of the basal levels of AEA in the horse. Due to this, in a similar manner to OEA and the 100 µL method, non-parametric statistics was completed on the AEA sample set. From this, a non-parametric workflow to determine the population reference limits with table 6.13 highlighting the estimated upper and lower thresholds. As this is in reference to non-parametric statistics, the original data set of 187 samples was used.

Table 6-13: Results from the threshold equation for AEA in equine plasma using the non-parametric statistics (n=187).

Calculations	Population Reference Limit (ng/mL)
Estimated upper threshold	0.67
Estimated lower threshold	0.12

From the non-parametric statistics, the upper and lower thresholds were estimated to be 0.67 ng/mL and 0.12 ng/mL. These concentrations appear to be more appropriate given the concentration range of AEA. The 100 µL method estimated upper and lower thresholds of 0.6 ng/mL and 0.2 ng/mL (the LOQ). Whilst the lower threshold is similar, the upper threshold is slightly lower which could be attributed to the larger sample volume being analysed using the routine method. Likewise, with OEA, cooperation with other interstate and international laboratories would help determine if the concentrations obtained in this study was consistent through their populations.

6.4.5 Equine Biological Passport - Individual Reference Limits

The EBP as previously stated is considered an intelligence model to longitudinally monitor specific horses and their individual levels of biomarkers present in each equine system. As an individual horse's concentration of marker could be different to a general population threshold, it is important to establish the profile for each horse. This may include horses that have levels of endogenous compound which may exceed the threshold due to natural variation. Using equations 2.8 to 2.11 in chapter 2.2.10, horses 10 and 11 from the EBP have been chosen to be profiled using the routine method.

The HC/C ratio had the ability to generate longitudinal profiles this data has been collected for almost 5 years. Previous work focusing on the HC/C ratio detailed there is no significant difference between the three genders of horses, therefore, the combined reference population statistics was utilised[93].

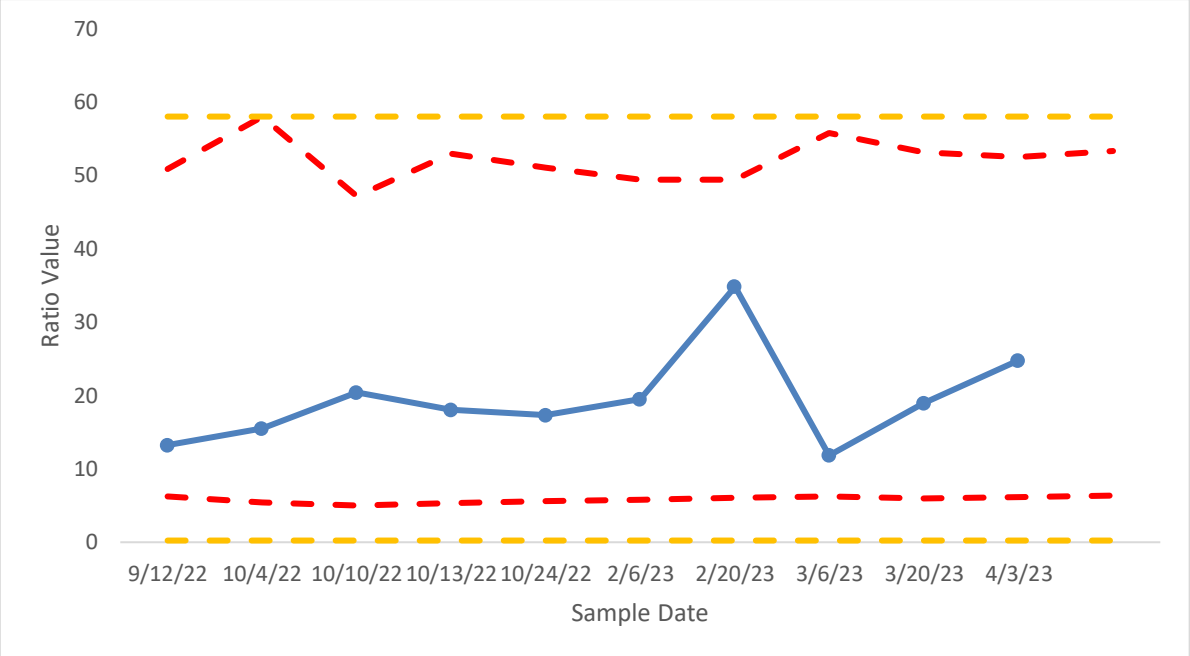

Figure 6.14: Intra-individual profile for Horse 10 using HC/C ratio (Red lines indicate upper and lower IRLs, yellow lines indicate upper and lower PRLs, and blue line indicates the ratio).

As seen in horse 10 in Figure 6.14, there appears to be no abnormal results exceeding either the upper or lower IRL. The ratio values do not exceed either the upper (58) or lower (0.24) thresholds derived from the reference population. There appears to be an increase of the HC/C ratio from the sample on the 20th of February 2023 at a ratio of 35 however, as the ratio for this specific horse started lower at 13, the increase in HC/C was not deemed abnormal by either upper or lower IRL or PRL. The ratio then decreases by the next sample on the 6th of March 2023 but is slowly increasing again by the last sample on the 3rd of April 2023. Unfortunately, sampling for this horse had stopped after this point due to time restrictions on this project, so it is unknown if this horse's ratio values continued to increase or if the horse had continued racing after the 3rd of April. With more sampling, this profile would ultimately gain more insight into the IRL for this horse's ratio and individual level of the HC/C ratio.

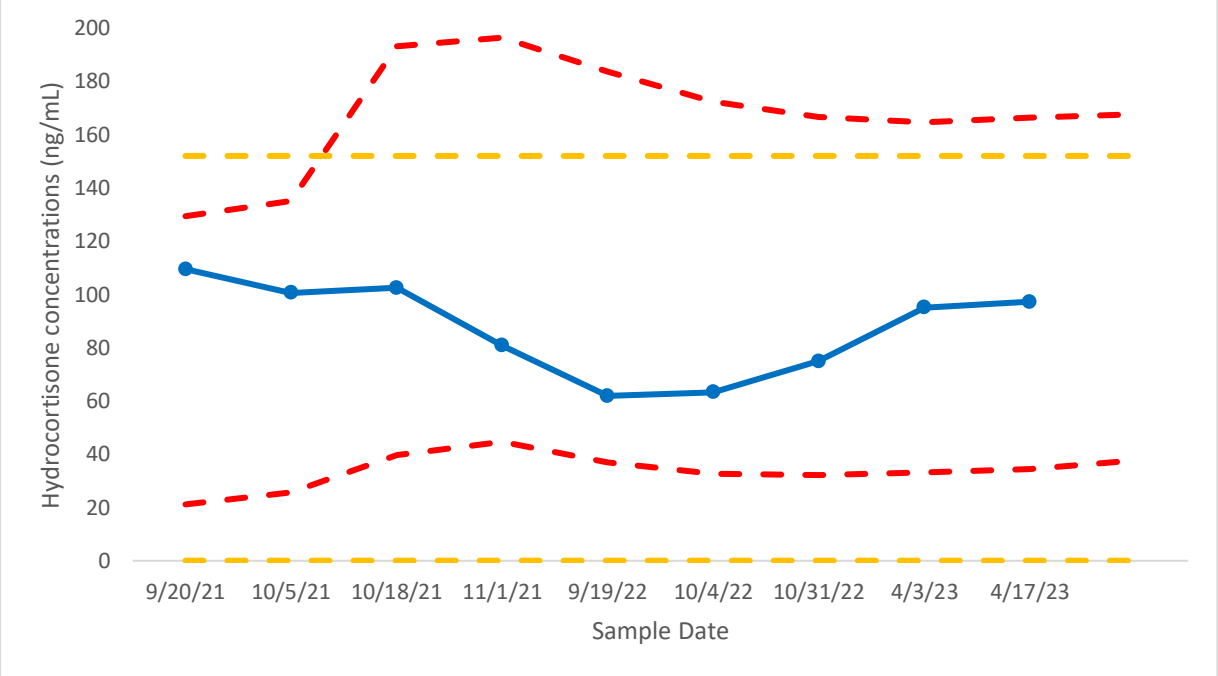

Figure 6.15: Intra-individual profile for Horse 11 using hydrocortisone only (Red lines indicate upper and lower IRLs, yellow lines indicate upper and lower PRLs, and blue line indicates the ratio).

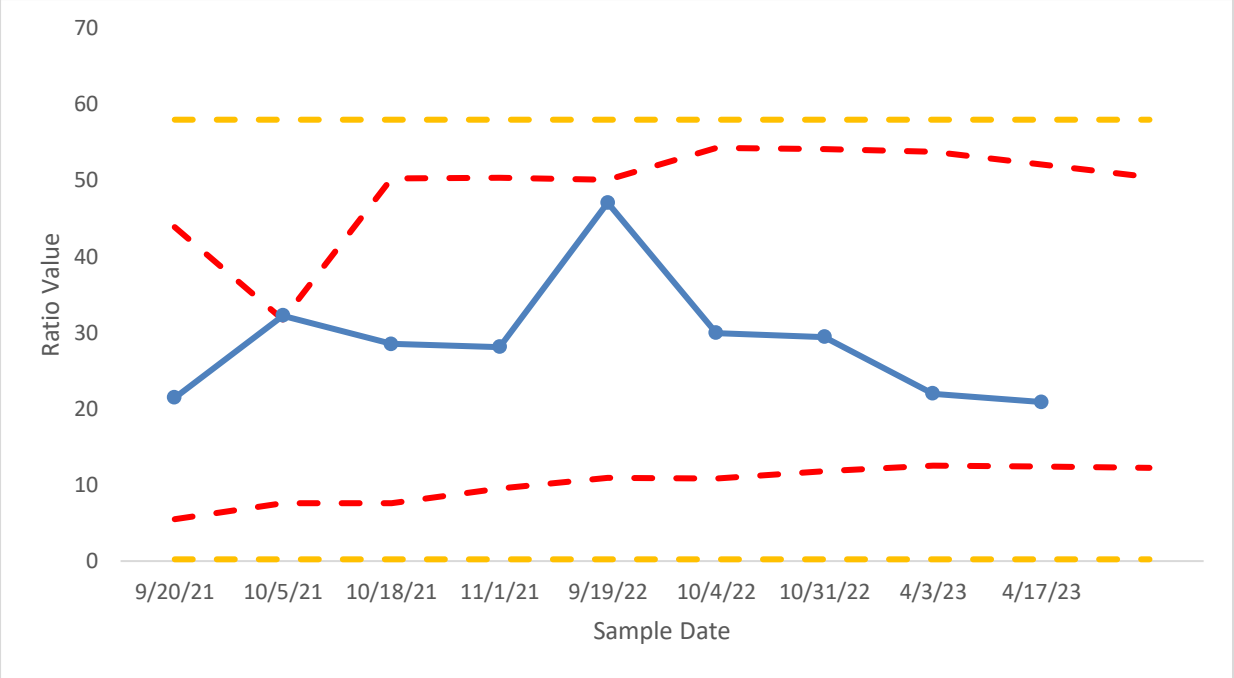

Figure 6.16: Intra-individual profile for Horse 11 using HC/C ratio (Red lines indicate upper and lower IRLs, yellow lines indicate upper and lower PRLs, and blue line indicates the ratio).

As seen in Figure 6.15, the profile for Horse 11 showed no abnormal results for hydrocortisone with no exceedance of either IRL or the proposed PRL of 152 ng/mL and 0.04 ng/mL for upper and lower respectively. The IRL does appear to exceed the PRL for this specific horse indicating a horse with higher hydrocortisone levels. Using the HC/C ratio for horse 11 however, as seen in Figure 6.16, whilst there is no sample that is deemed abnormal, the sample collected on the 19th of September 2022 is approaching the upper IRL with a value of 47 and the IRL of 50. As the sample on the 4th of October 2023 is approximately the same ratio as the sample on the 11th of November 2021, the sample from the 19th of September 2022 can be deemed suspicious considering how close it is to the upper IRL. This horse is a very good example of why certain horses will need monitoring at an individual level compared to the general population. Whilst this horse did have 9 samples, with more samples, the profile will improve for better longitudinal profiling over an extended period of time. This horse also demonstrates the benefit of using the ratio compared to just hydrocortisone itself. Nevertheless, the sample from the 19th of September 2022 could be deemed as intelligence

with information being provided to stewards for further investigation (e.g., review of veterinary treatment records).

Therefore, from this study of using the IRLs, more work is definitely required to improve the profiling for each individual horse considering the abnormalities seen within the profiles. Firstly, with more sampling, the algorithm would be able to become more weighted towards the individual horse's individual levels of hydrocortisone and the ratio rather than relying on the general population. Secondly, as the equations were proposed by McIntosh for IRLs, this may need further refinement in the future to possibly detect for more abnormalities while retaining a high degree of confidence. The major limitations of these profiles are the careers of racehorses are short-lived in comparison to the athlete biological passport where samples are plentiful. Ideally in the future, the parametric empirical bayes algorithm can be altered to accommodate for the sample limitations. Further investigations are also required for a non-parametric model as this model heavily relies on the use of parametric only biomarkers. With a non-parametrical model, this could improve the scope of the use of IRLs to nearly any biomarker.

6.5 Research to Routine Conclusion

The necessity to effectively translate a research method to a routine analysis method is extremely important to ensure the research can be utilised to further expand the scope of detection for each laboratory. The research method of 100 µL of equine plasma for the detecting of lipid and corticosteroid biomarkers was appropriately translated to the routine 2 mL extraction method of equine plasma for one lipid biomarker, OEA. The biomarker of AEA, however, did not pass criteria for accuracy and as such, concentrations for AEA were deemed as estimations only. These two biomarkers are in addition to the previously identified and validated corticosteroid biomarkers of hydrocortisone, cortisone, and the HC/C ratio. OEA and AEA were method validated under the criteria of specificity, selectivity, linearity, precision, recovery, matrix effects and stability. The LOD and LOQ for OEA and AEA were estimated to be < 0.01 ng/mL and 0.02 ng/mL respectively. Accuracy for AEA and recovery for both AEA and OEA were low likely due to the use of sub-optimal method for the extraction of

the lipid biomarkers. Stability studies showed predominately a 1-month period of stability for the lipid biomarkers.

Using the reference population data collated for this project, boxplots showed no significant difference between the genders for the lipid biomarkers indicating the possibility of proposing a threshold that can be non-gender specific. The lipid biomarkers indicated a parametric distribution of 89.7% for OEA and 73% for AEA. However, as the parametric statistics provided inappropriate upper and lower thresholds for OEA and AEA, non-parametric statistics were completed to propose the reference population thresholds. Therefore, for OEA, 5.5 ng/mL was proposed for the upper threshold and 0.02 ng/mL for the lower threshold. For AEA, 0.67 ng/mL was proposed for the upper threshold and 0.12 ng/mL for the lower threshold.

The profiles for HC/C using the routine method could however be generated due to the ongoing study for the last 5 years. Horse 10 and 11 were utilised with horse 10 showing no abnormalities for a 1 ½ year period but horse 11 showed a sample approaching the upper IRL which would be deemed suspicious utilising the HC/C ratio but not hydrocortisone itself. These types of abnormal samples would undergo further investigation to determine if there was a reasoning for the samples to approach the IRL. The profile of horse 11 highlights the advantages of using ratios in comparison to biomarkers alone.

The biomarkers of OEA and AEA ideally would be added into the existing routine method with the addition of the OEA-D_4 internal standard. A successful validation of AEA quantification will also need to be completed; however, a significant number of resources are required before the addition of this biomarker. The addition of OEA and AEA will allow for the additional monitoring of these biomarkers without having the need to utilise the 100 µL method saving time and resources. The addition of these two markers will also allow for additional intelligence that an exogenous administration was given as seen from previous chapters. However, international collaboration with other laboratories would need to be completed prior to these biomarkers being added to ensure the levels being detected are consistent throughout different environments. The EBP has heavily benefitted from the research to routine method with now the possibility of an additional 2 biomarkers not previously researched. Further work is necessary to include the 2 lipid biomarkers for longitudinal profiling to allow for a more individual understanding of OEA and AEA.

Research to Routine References

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Chapter 7:
Conclusions and Future Work

7.1 Overall Conclusions

The use of lipidomics has allowed for a comprehensive yet novel study of the lipid pathways to complement existing methods for the equine anti-doping industry. The development of additional analytical methods for the analysis of eicosanoids (especially those involved in inflammation) is needed due to the integrity and ethical issues arising for the therapeutic use but also misuse of anti-inflammatory substances. This project has allowed for a better understanding of the lipid and corticosteroid biomarkers in the equine system and how these biomarkers are affected by the administration of corticosteroids and bisphosphonates.

A diverse range of 20 lipids and 5 corticosteroids were investigated for targeted analysis. From these targeted analytes, OEA and C were validated for full quantification whilst AEA and HC were partially validated allowing for qualitative analysis using a 100 µL sample method. AEA and HC provided estimated concentrations whilst OEA and C were fully quantified using equine plasma from 750 pre-race blood samples collected by Racing NSW to determine basal concentrations. In agreement with previously published literature, the concentrations of C were relatively consistent not exceeding 13 ng/mL, allowing for its use as an ERC in the previously proposed HC to C ratio. Table 7.1 summarises the upper and lower proposed population reference limits (PRLs) using parametric statistics and non-parametric statistics.

Table 7-1: Proposed upper and lower PRLs for 100 µL method. Concentrations of AEA and HC are deemed as estimations.

Compound	Parametric, non-parametric statistics or estimated range	Upper proposed reference limit	Lower proposed reference limit
AEA	Estimated range	0.6 ng/mL	0.2 ng/mL
OEA	Non-parametric	5.6 ng/mL	0.21 ng/mL
HC	Parametric	200 ng/mL	0.2 ng/mL
C	Parametric	10 ng/mL	1.0 ng/mL
HC/C	Parametric	50	0.12

The use of these levels with additional longitudinal profiling of horses on the Racing NSW EBP enabled evaluations over a 4-year period for the HC/C ratio between 2019 and 2022 as seen in chapter 2.5 and in previously published literature[93]. The EBP profiles highlight the advantages and disadvantages of using individual reference limits (IRLs) with atypical profiles being investigated further.

With an understanding of the basal levels for specific biomarkers, four administrations studies were conducted to investigate the effects on these compounds following 2 exogenous corticosteroid administrations and 2 bisphosphonate administrations. The first administration of TACA was assessed in relation to the biomarkers identifying an intra-articular administered corticosteroid within 8-clear days of racing. From this pilot TACA administration study, the parent drug was detectable up to 2.5 days post-administration. With the monitoring of the lipid and corticosteroid biomarkers: 15(S)-HETE, 5(S)-HETE, AA, PGD_2, $PGF_{2\alpha}$, AEA, HC and HC/C, complementary indirect detection was supported with effects observed up to 9 days post-administration. Therefore, in relation to the 8-clear day rule, the complementary use of biomarkers can better support the Rules of Racing. Further work is necessary to assess whether additional horse sampling would observe consistent results. The second corticosteroid administration was fluticasone propionate (FLP) by inhalation. This presents the challenge of low drug deposition in the lungs resulting in detection issues for plasma samples. The parent compound of FLP was not detectable using the developed method, therefore a complementary biomarker approach to provide indirect detection was assessed. OEA, estimated HC concentrations and HC/C values provided extended race day detection of FLP. In addition, the use of IRLs provided an additional 4 hours of intelligence period that an administration had taken place.

The two BP administrations of zoledronic acid and tiludronic acid yielded interesting results given the current lack of literature regarding the effects on the eicosanoids under the presence of BPs. The first aim of the BP administrations was successfully investigated with ZA detected up to 8 hours post-administration and TA detected for 28 days post-administration. The second aim of the BP administration was also successfully investigated with $PGF_{2\alpha}$, 15(S):5(S)-HETE ratio and 10-HDHA providing evidence of effect following TA administration. Similarly, exploring a ZA administration, the analytes of HC/C and DHA showed evidence of

effect allowing for the indirect extended detection of ZA from 8 hours to 28 days post-administration. With the different analytes being affected between a TA and ZA administration, this has allowed for the third aim to be successfully completed as the analytes were able to be differentiated between a non-nitrogenous and nitrogenous BP. It was also noted that OEA can provide evidence of a BP administration, however further investigation would be necessary for better determination of the specific BP.

Whilst the ability to detect for compounds in a research setting is important, effective translation of a research method into a routine analysis provides the best return-on-investment. In turn, this enables research to expand the scope of laboratory detection methods. From this work, the investigative 100 µL sample method was translated to a routine 2 mL sample method to determine if the selected 8 lipid and 3 corticosteroid biomarkers could be accurately quantified. This list of lipids and corticosteroid biomarkers were chosen from the change seen in the administrations discussed previously. OEA was successfully validated, in addition to previously validated corticosteroids HC, C and the HC/C values were successfully implemented in routine ARFL procedures. AEA did not satisfy accuracy criteria and therefore, concentrations were all deemed as estimations. Following non-parametric transformations, the following limits were proposed for AEA and OEA. (Table 7.2 summarised the proposed upper and lower limits for the routine method).

Table 7-2: Proposed upper and lower PRLs for 2 mL method. AEA concentrations are estimations only.

Compound	**Upper proposed reference limit (ng/mL)**	**Lower proposed reference limit (ng/mL)**
AEA	0.67	0.12
OEA	5.5	0.02

There are many benefits to longitudinally profile these lipid biomarkers, however due to limited retrospective sample analysis from the 2 mL sample method, profiles could not be appropriately generated. There is the potential for these markers to be added to the current EBP scope for a more comprehensive understanding of the equine response to anti-inflammatory and/or analgesic stimuli. The EBP benefits from this research with additional

biomarkers applied to the anti-doping setting. Estimated AEA and OEA values could be internationally recognised as biomarkers to monitor in addition to HC, C and the HC/C values for improved indirect detection of corticosteroid corticosteroids and bisphosphonate administrations.

7.2 Limitations

There have been many limitations which were encountered throughout this study. In the reference population study utilising the research 100 µL method, AEA could not be accurately or precisely quantitated not allowing for a full quantification and non-parametric statistical analysis to propose upper and lower thresholds. Furthermore, it was seen in the concentrations of OEA that a higher sensitivity is necessary to fully quantify the lower concentrations. Concentrations of HC were also deemed as estimations for the 100 µL method due to higher percent relative error however, for the 2 mL method, was within acceptable range for accuracy highlighting the need for more sample volume if the use of biomarkers was to ever be implemented routinely. For the routine 2 mL method reference population study, only 268 samples were analysed compared to 750 for the 100 µL method. This smaller set of samples could have affected the range of concentrations obtained.

For the TACA, FLP and BP administrations, a major limitation of this study is the lack of placebo treated horses in the experimental design. Due to this, it cannot be fully known whether the biomarker effects seen were due to the administration itself or from external factors. Specifically, for the TACA and FLP administrations, only 3 horses were utilised which limited the power calculations and sample size analysis. For the BP administrations, as ZA was administered 12 months prior to TA, it is unknown whether the ZA had long lasting effects which may have affected the results obtained from TA.

7.3 Recommendations for future work

As this research focused only on a targeted and semi-targeted list of eicosanoids, future work investigating other compounds following a different precursor cascade could extend the lipidomic scope for anti-doping purposes. Additionally, repeating the full method validation

for AEA to ensure criteria is met would allow for a more accurate quantification to be completed. It would also be beneficial to increase the number of samples utilised for the reference population using a routine method to capture the basal levels of the lipids across a larger number of horses. This would allow for a greater understanding of the natural variance within the population with the aim of establishing thresholds. The use of multiple biomarkers and/or their ratios provides greater confidence if there is a finding of a misuse of a doping agent rather than natural variation.

The potential of a threshold will rely on adherence to the Association of Official Racing Chemists (AORC) guidelines. Samples would need to be exchanged, extracted, and analysed from multiple international laboratories to capture environmental and seasonal variations, together with identifying laboratory bias. Once the various jurisdictions have completed their corresponding analyses, an international consensus would need to be accepted by other members of the AORC before the implementation of the threshold to be used in a routine setting.

Untargeted analysis of FLP administration needs to be conducted to examine whether there are any biomarkers not monitored through targeted and semi-targeted screening. With non-quantifiable concentrations of FLP, the use of biomarkers for indirect screening could provide significant advantages to ensure FLP misuse can be identified.

The use of IRLs allows for comparisons with the horse itself rather than the general population, allowing for the natural variation within the horse to be accounted for. Whilst the use of IRLs for intelligence purposes is advantageous, at present it is limited to only monitoring within a single laboratory. Therefore, for the passport to realise greater potential, the implementation into other racing laboratories would be beneficial in addition to investigating inter-laboratory variances. Such an implementation could be based on WADA's ABP. Furthermore, it could be beneficial to have machine learning algorithms to improve estimation of IRLs to reduce the number of false negatives results. Finally, the extension of using non-parametric approaches for estimating biomarker IRLs would be a further advance since not all compounds display a parametric distribution, thereby limiting the application of the PEB model. Implementation of non-parametric IRLs could substantially increase the number of EBP biomarkers used for intelligence purposes, thereby providing a more holistic

view of each individual horse. Further work would need to be completed to facilitate this with advice from a biostatistician necessary to ensure correct interpretation of the data. This would, in turn, expand the number of drug classes and doping agents within them covered by this intelligence-based strategy.

Conclusion References

93. Tou K, Cawley A, Bowen C, Sornalingam K, Fu S. Measurements of hydrocortisone and cortisone for longitudinal profiling of equine plasma by liquid chromatography-tandem mass spectrometry. *Drug Test Anal.* 2022;14(5):943-952.

Chapter 8: Appendices

Appendix 1: Endogenous Lipids and Corticosteroids

Instrument parameters for the LC-HRMS

Table A1: MS Conditions for LC-HRMS.

Conditions	Setting
Gas Interface Temperature (°C)	270
DL Temperature (°C)	250
Block Heater Temperature (°C)	400
Drying Gas Flow (L/min)	10
Heating Gas Flow (L/min)	10
Nebulising Gas Flow (L/min)	2.5

Method validation preparation parameters

Linearity

Table A2: Plasma (100 µL) spike calibration preparation summary for AEA, OEA, HC and C.

Spike Concentration (ng/mL)	Analyte Solution	Amount (µL)
0	-	-
1	Working 10 ng/mL	10
2	Working 10 ng/mL	20
5	Working 10 ng/mL	50
10	Working 10 ng/mL	100
50	Stock 2000 ng/mL	25
100	Stock 2000 ng/mL	50
200	Stock 2000 ng/mL	100

Sensitivity

Table A3: Plasma spike for LOD and LOQ preparation in 100 µL of equine plasma.

Spike Concentration (ng/mL)	Solution	Amount (µL)
0.1	Working 1 ng/mL	10
0.2	Working 1 ng/mL	20
0.3	Working 1 ng/mL	30
0.4	Working 1 ng/mL	40

Method validation results - linearity

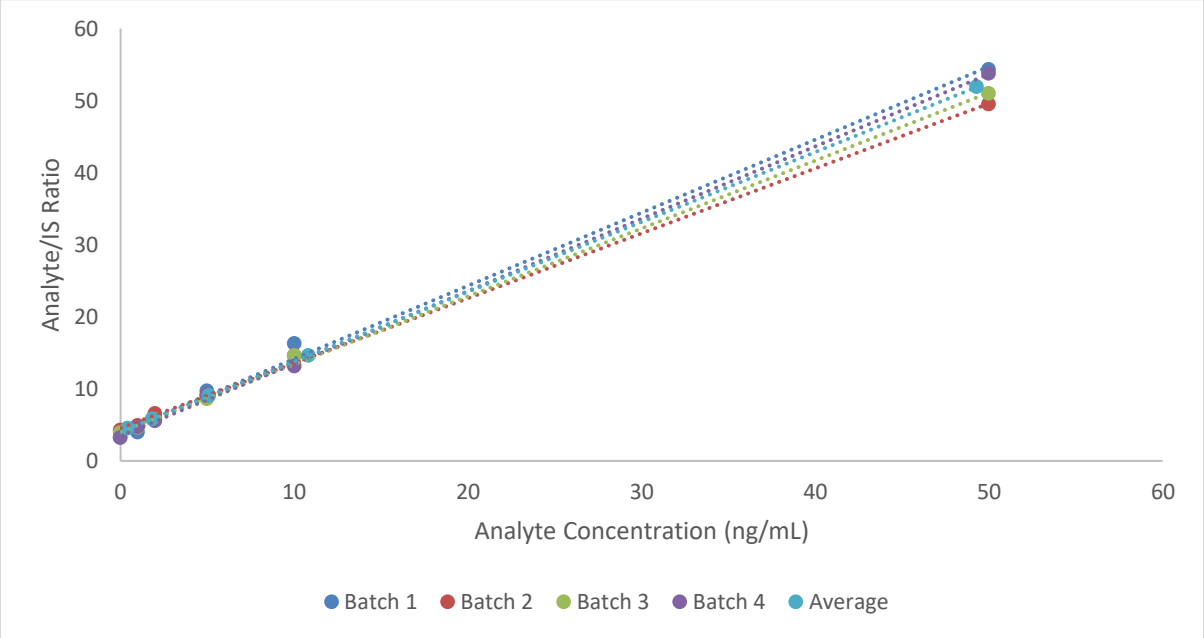

Figure A1: Linearity of OEA in equine plasma over 4 separate batches. Batch 1 equation: y = 1.0139x + 4.03 (R^2 = 0.9963), Batch 2 equation: y = 0.9009x + 4.5571 (R^2 = 0.999), Batch 3 equation: y = 0.9419 x + 4.004 (R^2 = 0.9991), Batch 4 equation: y = 1.0037 x + 3.4934 (R^2 = 0.9997), Average equation: y = 0.9698x + 4.069 (R^2 = 1)

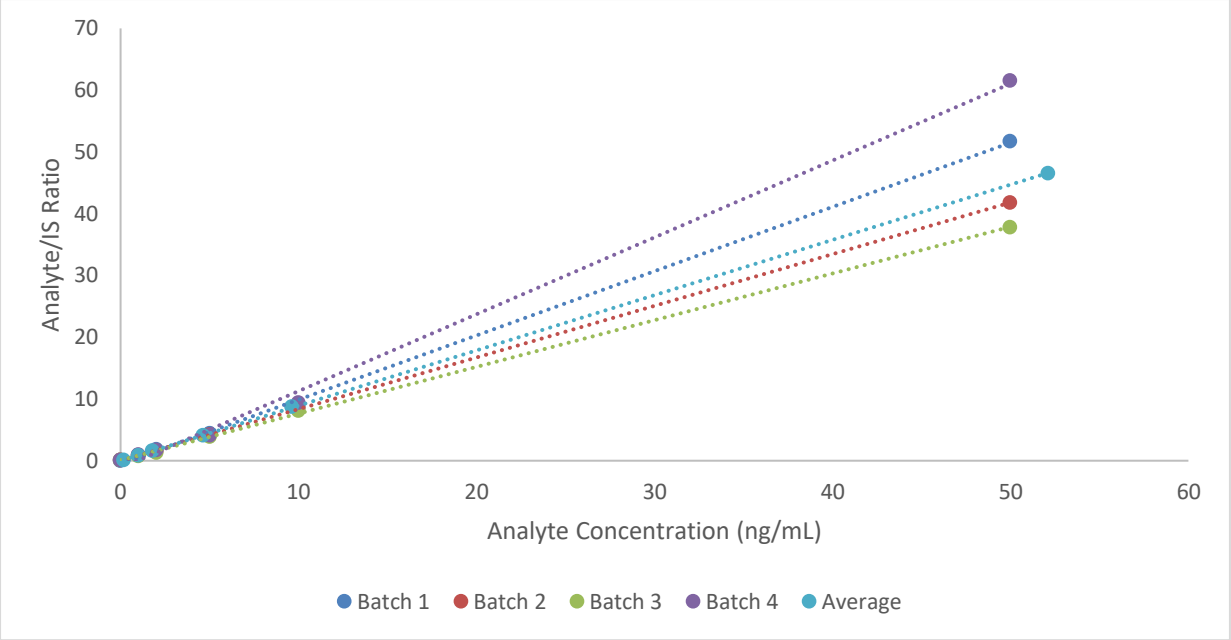

Figure A2: Linearity of AEA in equine plasma over 4 separate batches. Batch 1 equation: y = 1.0402x – 0.513 (R^2 = 0.9993), Batch 2 equation: y = 0.8365x – 0.0169 (R^2 = 1), Batch 3 equation: y = 0.7561 x + 0.0733 (R^2 = 0.9997), Batch 4 equation: y = 1.2446x – 1.1787 (R^2 = 0.9976), Average equation: y = 0.8945x – 0.026 (R^2 = 1)

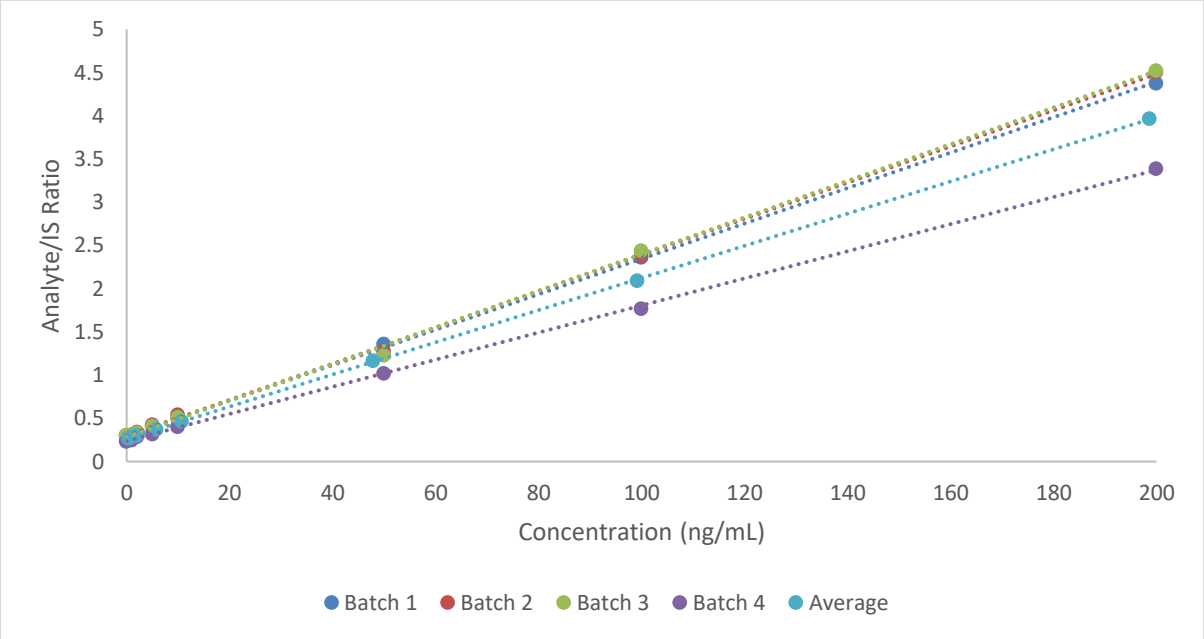

Figure A3: Linearity of hydrocortisone in equine plasma over 4 separate batches. Batch 1 equation: y = 0.0205x + 0.2964 (R^2 = 0.9998), Batch 2 equation: y = 0.0209x + 0.2949 (R^2 = 0.9995), Batch 3 equation: y = 0.0212x + 0.2807 (R^2 = 0.999), Batch 4 equation: y = 0.0157 x + 0.2376 (R^2 = 0.9998), Average equation: y = 0.0186x + 0.2631 (R^2 = 1)

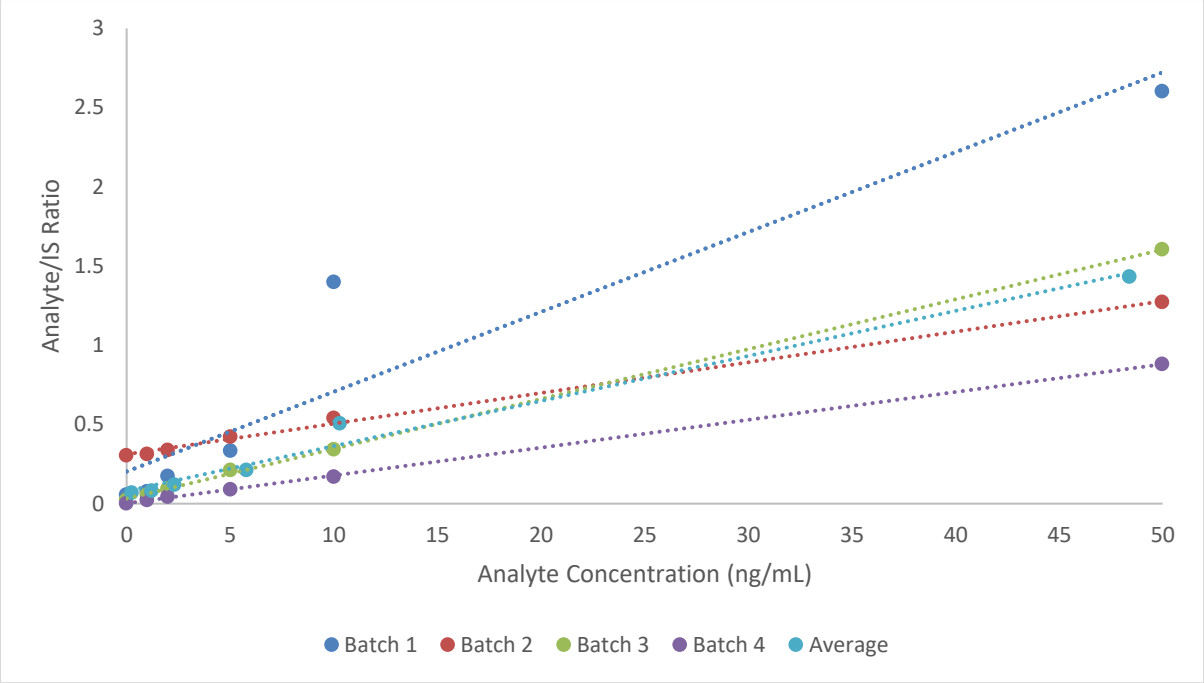

Figure A4: Linearity of cortisone in equine plasma over 4 separate batches. Batch 1 equation: 0.0504x + 0.2026 (R^2 = 0.8907), Batch 2 equation: y = 0.0193x + 0.3117 (R^2 = 0.9971), Batch 3 equation: y = 0.0314x + 0.0318 (R^2 = 0.9997), Batch 4 equation: y = 0.0176x + 0.0022 (R^2 = 0.9998), Average equation: y = 0.0284x + 0.0793 (R^2 = 0.9847)

Appendix 2: Triamcinolone acetonide administration

Method validation preparation parameters

Table A4: Plasma (100 µL) spike calibration preparation summary for TACA.

Spike Concentration (ng/mL)	Analyte Solution	Amount (µL)
0	-	-
0.05	Working 0.5 ng/mL	10
0.1	Working 0.5 ng/mL	20
0.2	Working 0.5 ng/mL	40
0.5	Working 1 ng/mL	50
1	Working 10 ng/mL	10
2	Working 10 ng/mL	20
5	Working 10 ng/mL	50

TACA Results

Table A5: Acquired Data for targeted lipid analytes for TACA, 15-HETE and 5-HETE for all 3 horses. All TACA concentrations and any biomarker concentrations over 200 ng/mL are deemed estimates. (Not detected = ND).

Analyte	TACA (ng/mL)			15-HETE (ng/mL)			5-HETE (ng/mL)		
Horses	Horse 1	Horse 2	Horse 3	Horse 1	Horse 2	Horse 3	Horse 1	Horse 2	Horse 3
-1 Day	ND	ND	ND	36.9	76.7	68.7	232	542	187
T0	ND	ND	ND	19.9	26.1	58.1	89.1	312	122
2 min	ND	0.115	ND	25.1	18.1	58.7	118	162	236
5 min	0.200	0.745	ND	19.9	19.6	65.9	90.0	76.5	294
10 min	0.700	1.17	0.420	20.7	24.5	50.5	129	86.7	177
20 min	1.02	1.53	1.27	19.5	20.3	63.4	94.1	117	152
30 min	1.04	1.38	0.930	18.1	18.1	88.8	72.5	270	136
40 min	0.626	1.24	0.840	24.2	27.3	57.2	114	90.6	171
50 min	0.486	1.21	0.744	22.7	20.1	67.2	118	149	96.6
60 min	0.596	1.12	0.615	22.5	39.5	51.1	113	190	150
90 min	0.844	1.92	0.623	17.9	22.3	52.9	96.2	156	80.0
2 h	0.605	1.72	0.763	18.6	20.4	43.6	89.6	101	196
2.5 h	0.387	2.07	1.12	26.1	20.1	57.9	179	128	65.9
3 h	0.890	2.58	1.55	22.4	22.6	64.5	115	141	128
4 h	1.27	2.42	1.42	25.7	29.2	71.6	250	161	70.8
6 h	1.10	2.03	1.42	34.0	28.2	74.0	215	192	146
8 h	1.02	1.64	1.51	29.4	29.0	50.0	262	165	75.0
12 h	0.709	1.54	1.80	33.2	24.5	61.5	212	135	166
24 h	0.343	0.864	1.11	21.9	26.4	18.7	120	425	113
36 h	0.144	0.512	0.392	27.5	26.8	72.3	208	180	163
48 h	ND	0.253	0.107	21.5	28.3	52.7	94.3	187	85.1

60 h	0.0220	0.300	0.230	27.3	27.0	76.8	109	323	166
72 h	ND	0.0580	ND	38.6	25.0	64.0	221	156	175
96 h	ND	0.00200	ND	26.4	32.0	65.8	201	264	257
120 h	ND	ND	ND	27.2	20.8	67.6	133	86.2	155
144 h	ND	ND	ND	22.3	30.7	91.9	109	159	211
168 h	ND	ND	ND	21.3	23.8	8.33	117	255	91.8
192 h	ND	ND	ND	27.9	42.5	74.3	162	148	111
216 h	ND	ND	ND	35.2	23.4	79.8	152	336	307

Table A6: Acquired Data for targeted lipid analytes for AA for a TACA administration for all 3 horses. All concentrations are deemed estimates.

Analyte	**AA (ng/mL)**		
Horses	Horse 1	Horse 2	Horse 3
-1 Day	9.99E+02	5.61E+02	1.62E+03
T0	1.12E+03	6.35E+02	1.55E+03
2 min	9.42E+02	4.81E+02	1.47E+03
5 min	1.04E+03	5.42E+02	1.65E+03
10 min	1.02E+03	6.49E+02	1.52E+03
20 min	1.03E+03	5.89E+02	1.52E+03
30 min	1.06E+03	5.25E+02	1.49E+03
40 min	1.29E+03	6.53E+02	2.42E+03
50 min	1.26E+03	5.28E+02	1.59E+03
60 min	1.08E+03	6.21E+02	1.35E+03
90 min	1.21E+03	6.26E+02	1.23E+03
2 h	1.09E+03	6.84E+02	1.79E+03
2.5 h	1.03E+03	6.28E+02	1.39E+03
3 h	1.10E+03	5.80E+02	1.69E+03
4 h	1.20E+03	5.93E+02	1.61E+03
6 h	1.10E+03	5.15E+02	1.43E+03

8 h	1.02E+03	4.96E+02	1.12E+03
12 h	1.03E+03	5.15E+02	1.21E+03
24 h	8.48E+02	6.19E+02	1.13E+03
36 h	1.03E+03	5.16E+02	1.66E+03
48 h	1.11E+03	4.90E+02	1.12E+03
60 h	1.02E+03	4.75E+02	1.13E+03
72 h	1.18E+03	5.12E+02	1.41E+03
96 h	1.12E+03	5.49E+02	1.52E+03
120 h	1.11E+03	5.14E+02	1.75E+03
144 h	1.10E+03	5.15E+02	1.93E+03
168 h	1.32E+03	5.84E+02	8.72E+02
192 h	1.02E+03	6.82E+02	1.61E+03
216 h	1.06E+03	6.34E+02	1.27E+03

Table A7: Acquired Data for targeted lipid analytes for PGD_2 and $PGF_{2\alpha}$ for a TACA administration for all 3 horses. All concentrations are deemed estimates. (Not detected = ND).

Analyte	**PGD_2 (ng/mL)**			**$PGF_{2\alpha}$ (ng/mL)**		
Horses	Horse 1	Horse 2	Horse 3	Horse 1	Horse 2	Horse 3
-1 Day	3.91	7.84	1.08	0.411	1.26	1.14
T0	0.909	1.29	0.747	0.170	0.963	1.21
2 min	1.97	0.710	0.875	0.361	0.767	1.20
5 min	0.520	1.06	0.345	0.0410	0.882	0.943
10 min	0.883	0.511	0.413	0.167	0.809	1.13
20 min	0.818	0.881	0.357	0.179	0.815	1.01
30 min	0.193	0.817	13.8	ND	0.645	1.14
40 min	1.29	1.45	0.531	0.255	0.961	1.20
50 min	1.01	1.93	1.25	0.0450	0.922	1.20
60 min	1.93	2.26	0.507	0.277	1.08	0.912

90 min	0.325	1.27	1.41	0.0350	0.815	1.09
2 h	0.709	1.11	0.105	ND	0.675	0.906
2.5 h	2.60	0.994	1.59	0.197	0.869	1.04
3 h	1.17	1.06	1.57	0.122	0.765	1.13
4 h	1.70	0.985	1.25	0.132	0.847	1.57
6 h	3.28	1.60	1.23	0.488	1.05	1.56
8 h	2.28	1.06	1.03	0.317	0.998	1.31
12 h	2.97	0.753	4.34	0.427	0.801	1.33
24 h	1.15	0.765	3.77	0.252	1.03	1.49
36 h	1.26	0.366	3.70	0.245	1.12	1.58
48 h	0.345	0.636	0.699	0.138	1.01	1.07
60 h	0.364	0.983	0.761	0.132	0.998	1.06
72 h	1.27	0.976	0.849	0.284	0.851	1.18
96 h	0.296	1.10	0.439	0.100	1.03	1.06
120 h	1.45	0.902	0.836	0.137	0.743	0.982
144 h	ND	0.943	0.749	0.036	0.867	1.13
168 h	0.421	0.616	0.752	0.00200	0.770	1.33
192 h	0.794	1.26	0.470	0.174	0.932	1.14
216 h	1.39	0.460	0.331	0.149	0.705	1.07

Table A8: Acquired data for targeted lipid analytes for AEA and OEA for a TACA administration for all 3 horses. AEA concentrations are deemed estimates. (Not Detected = ND).

Analyte	**AEA (ng/mL)**			**OEA (ng/mL)**		
Horses	Horse 1	Horse 2	Horse 3	Horse 1	Horse 2	Horse 3
-1 Day	0.190	0.220	0.264	5.45	4.64	3.31
T0	0.128	0.193	0.219	1.44	0.0260	0.700
2 min	0.137	0.188	0.246	2.21	1.41	1.79
5 min	0.130	0.199	0.252	1.56	1.29	0.555

10 min	0.127	0.200	0.237	1.60	1.61	1.16
20 min	0.129	0.210	0.227	1.24	1.38	1.48
30 min	0.138	0.199	0.228	1.94	1.50	1.52
40 min	0.126	0.189	0.269	1.23	0.792	1.20
50 min	0.126	0.200	0.235	1.14	1.19	1.01
60 min	0.126	0.204	0.240	1.16	0.566	2.07
90 min	0.139	0.213	0.264	1.75	2.33	2.36
2 h	0.165	0.241	0.313	3.16	3.91	3.09
2.5 h	0.174	0.240	0.368	3.91	4.09	4.22
3 h	0.181	0.249	0.291	4.58	3.36	1.89
4 h	0.142	0.210	0.259	3.91	2.59	1.87
6 h	0.121	0.175	0.217	1.50	0.234	1.32
8 h	0.122	0.189	0.214	0.848	0.196	0.794
12 h	0.131	0.171	0.219	1.03	0.109	ND
24 h	0.137	0.186	0.191	1.64	0.600	ND
36 h	0.127	0.169	0.216	0.321	ND	ND
48 h	0.134	0.184	0.260	2.82	0.791	3.00
60 h	0.107	0.176	0.204	2.40	0.906	2.14
72 h	0.121	0.186	0.237	3.62	2.02	3.57
96 h	0.128	0.191	0.236	5.72	3.15	2.84
120 h	0.136	0.219	0.254	6.01	4.76	6.42
144 h	0.145	0.180	0.187	4.52	2.03	1.93
168 h	0.137	0.181	0.229	5.12	3.38	2.08
192 h	0.124	0.185	0.189	4.68	4.79	2.56
216 h	0.140	0.177	0.198	5.48	4.37	2.18

Table A9: Acquired TACA Data for targeted corticosteroid analytes for 3 horses (Not detected = ND). HC concentrations are deemed estimates.

Analyte	**HC**			**C**			**HC/C**		
Horses	Horse 1	Horse 2	Horse 3	Horse 1	Horse 2	Horse 3	Horse 1	Horse 2	Horse 3
-1 Day	29.6	31.5	22.6	2.13	1.88	0.889	13.9	16.8	25.4
T0	47.5	38.2	13.7	2.24	1.66	1.06	21.2	23.1	12.9
2 min	55.8	41.2	32.5	2.44	1.71	0.484	22.8	24.1	67.1
5 min	49.4	38.9	34.6	2.34	1.61	0.582	21.1	24.1	59.5
10 min	49.9	46.5	29.3	2.52	1.50	0.762	19.8	31.0	38.5
20 min	46.6	55.1	27.3	2.25	1.49	1.41	20.7	37.1	19.3
30 min	43.2	37.8	24.3	2.28	1.66	1.24	18.9	22.8	19.7
40 min	40.6	36.3	19.3	2.18	1.96	1.13	18.6	18.5	17.1
50 min	37.0	35.8	18.5	2.06	1.85	1.34	18.0	19.3	13.8
60 min	31.8	29.9	15.8	1.97	1.86	0.964	16.2	16.1	16.4
90 min	21.5	21.4	9.59	1.72	1.46	0.949	12.5	14.7	10.1
2 h	16.7	14.5	3.97	1.43	1.21	0.725	11.7	12.0	5.48
2.5 h	12.6	7.92	ND	1.42	0.914	0.328	8.91	8.66	ND
3 h	6.76	2.67	ND	1.13	0.625	0.323	6.00	4.28	ND
4 h	1.95	ND	ND	1.08	0.324	0.102	1.80	ND	ND
6 h	ND	ND	ND	0.756	0.0500	ND	ND	ND	ND
8 h	ND	ND	ND	0.638	ND	ND	ND	ND	ND
12 h	ND	ND	ND	0.625	ND	ND	ND	ND	ND
24 h	ND	ND	ND	0.511	ND	ND	ND	ND	ND
36 h	ND	ND	ND	0.424	ND	ND	ND	ND	ND
48 h	ND	ND	ND	0.630	ND	ND	ND	ND	ND
60 h	ND	ND	ND	0.738	ND	ND	ND	ND	ND
72 h	ND	ND	ND	1.06	ND	ND	ND	ND	ND
96 h	10.1	ND	ND	1.36	ND	ND	7.41	ND	ND

120 h	11.6	ND	ND	1.29	ND	ND	8.95	ND	ND
144 h	47.9	ND	ND	1.82	ND	ND	26.3	ND	ND
168 h	38.7	ND	ND	2.09	ND	ND	18.5	ND	ND
192 h	63.1	ND	ND	2.58	ND	0.0560	24.5	ND	ND
216 h	60.4	ND	23.1	2.84	ND	0.722	21.3	ND	32.0

Table A10: Acquired TACA Data for semi-targeted analytes for 12-HETE, 13-HDHA and 17-HEDE for 3 horses. All concentrations are deemed estimates.

Analyte	**12-HETE (ng/mL)**			**13-HDHA (ng/mL)**			**17-HDHA (ng/mL)**		
Horses	Horse 1	Horse 2	Horse 3	Horse 1	Horse 2	Horse 3	Horse 1	Horse 2	Horse 3
-1 Day	145	508	297	4.74	7.08	11.2	4.77	7.21	11.2
T0	70.3	236	248	2.53	4.00	11.3	5.92	4.00	11.3
2 min	64.1	148	337	3.35	2.61	13.5	2.79	2.61	13.5
5 min	47.5	56.1	404	2.88	3.33	10.2	3.47	3.13	10.2
10 min	72.9	71.9	227	3.63	3.28	8.81	4.11	3.28	8.81
20 min	58.5	123	258	2.72	4.07	11.5	3.69	4.08	11.5
30 min	45.4	186	525	2.40	3.06	11.4	3.24	3.07	12.4
40 min	93.8	127	222	2.81	2.12	10.7	3.50	4.88	10.7
50 min	74.6	125	270	3.24	3.79	12.7	5.97	3.93	12.7
60 min	85.6	223	292	3.29	5.45	10.1	7.29	5.45	10.1
90 min	65.3	181	194	2.27	2.84	10.9	3.68	3.02	10.9
2 h	52.6	56.2	383	2.68	2.36	7.81	2.36	2.36	7.81
2.5 h	113	65.5	255	2.92	2.71	13.9	3.25	2.99	13.9
3 h	62.7	108	263	2.67	3.85	13.0	2.92	3.85	11.7
4 h	131	95.7	333	2.87	2.80	13.3	3.05	2.81	13.3
6 h	128	132	265	3.31	4.22	9.65	4.07	4.22	9.65
8 h	140	198	224	3.29	3.43	10.8	2.98	3.43	10.8
12 h	133	141	308	3.29	2.54	9.28	3.68	2.55	9.28

24 h	60.0	424	205	2.98	3.47	4.50	3.23	3.47	4.50
36 h	120	132	307	3.88	4.46	11.0	4.29	4.46	11.0
48 h	65.7	223	219	3.05	5.17	8.49	5.26	5.17	8.49
60 h	71.6	276	261	3.27	4.36	9.34	3.47	4.36	9.34
72 h	142	122	340	4.33	5.23	13.5	4.57	5.24	13.5
96 h	120	304	410	2.92	3.55	11.9	3.65	3.55	11.9
120 h	83.2	39.5	342	2.42	2.73	11.8	3.09	2.73	11.8
144 h	56.9	188	359	2.36	2.57	12.0	3.40	2.57	12.0
168 h	59.9	537	119	1.50	2.44	1.86	2.47	2.44	1.60
192 h	102	106	395	3.26	3.21	10.4	3.80	3.21	10.4
216 h	112	381	569	3.07	2.92	8.25	3.62	2.92	8.25

Table A11: Acquired TACA data for semi-targeted analyte 18-HEPE for 3 horses. All concentrations are deemed estimates.

Analyte	**18-HEPE (ng/mL)**		
Horses	Horse 1	Horse 2	Horse 3
-1 Day	232	19.3	28.3
T0	89.1	6.99	24.7
2 min	118	3.31	23.2
5 min	90.0	3.95	26.1
10 min	129	4.70	20.6
20 min	94.1	4.16	22.7
30 min	72.5	3.91	59.9
40 min	114	6.05	25.0
50 min	118	4.63	26.9
60 min	113	7.81	23.1
90 min	96.2	4.49	20.4
2 h	89.6	4.53	17.7
2.5 h	179	4.67	27.4

3 h	115	5.01	29.2
4 h	250	6.97	30.9
6 h	215	7.29	30.1
8 h	262	6.43	23.4
12 h	212	5.52	32.4
24 h	120	6.62	20.3
36 h	208	6.75	32.1
48 h	94.3	6.77	20.8
60 h	109	5.84	24.3
72 h	221	6.28	26.9
96 h	201	6.25	30.5
120 h	133	5.62	29.0
144 h	109	5.73	31.6
168 h	117	5.24	10.6
192 h	162	8.39	26.6
216 h	152	4.81	28.3

Table A12: Acquired TACA Data for semi-targeted analytes for 15-HEDE and 9-HOTrE for 3 horses (Note that peak area was used instead of concentration. For 15-HEDE, this was due to unquantifiable concentrations from high endogenous levels and for 9-HOTrE, this was due to high estimated concentrations obtained surpassing the highest calibrator).

Analyte	**15-HEDE**			**9-HOTrE**		
Horses	Horse 1	Horse 2	Horse 3	Horse 1	Horse 2	Horse 3
-1 Day	2.26E+04	9.24E+04	4.36E+04	6.08E+05	3.51E+05	2.64E+05
T0	1.10E+04	1.76E+04	5.25E+04	7.88E+05	3.58E+05	6.95E+05
2 min	1.28E+04	1.31E+04	4.09E+04	7.53E+05	3.64E+05	6.30E+05
5 min	1.18E+04	2.71E+04	3.97E+04	8.27E+05	4.24E+05	6.55E+05
10 min	1.43E+04	2.15E+04	3.34E+04	8.79E+05	5.00E+05	6.15E+05
20 min	1.42E+04	2.10E+04	3.50E+04	7.11E+05	4.76E+05	6.84E+05

30 min	1.02E+04	1.71E+04	8.17E+04	5.83E+05	4.39E+05	6.97E+05
40 min	1.48E+04	2.94E+04	3.71E+04	5.18E+05	4.61E+05	6.20E+05
50 min	1.45E+04	4.87E+04	4.62E+04	3.50E+05	3.81E+05	5.48E+05
60 min	1.56E+04	3.66E+04	4.43E+04	4.89E+05	3.87E+05	6.42E+05
90 min	8.74E+03	1.54E+04	5.02E+04	4.71E+05	3.39E+05	4.80E+05
2 h	8.06E+03	1.42E+04	2.57E+04	2.21E+05	2.77E+05	3.85E+05
2.5 h	1.19E+04	2.47E+04	3.48E+04	1.07E+05	2.74E+05	3.62E+05
3 h	8.97E+03	1.42E+04	4.67E+04	1.39E+05	1.79E+05	2.75E+05
4 h	1.04E+04	1.87E+04	3.73E+04	1.31E+06	9.05E+05	1.14E+06
6 h	1.39E+04	1.91E+04	3.16E+04	1.38E+06	1.28E+06	1.10E+06
8 h	1.20E+04	1.90E+04	3.12E+04	1.13E+06	1.54E+06	1.08E+06
12 h	1.31E+04	1.45E+04	2.69E+04	1.34E+06	1.26E+06	8.41E+05
24 h	1.13E+04	2.02E+04	1.10E+04	7.32E+05	5.58E+05	7.37E+05
36 h	1.64E+04	2.15E+04	3.75E+04	1.19E+06	1.18E+06	8.25E+05
48 h	1.11E+04	2.62E+04	2.94E+04	6.37E+05	6.61E+05	3.90E+05
60 h	1.07E+04	1.95E+04	2.30E+04	5.43E+05	1.02E+06	2.69E+05
72 h	1.82E+04	2.37E+04	3.06E+04	2.14E+05	2.31E+05	2.82E+05
96 h	1.13E+04	1.90E+04	3.60E+04	3.41E+05	4.62E+05	5.81E+05
120 h	1.04E+04	1.49E+04	2.68E+04	2.43E+05	2.39E+05	4.97E+05
144 h	8.99E+03	1.53E+04	3.69E+04	7.84E+05	5.84E+05	8.74E+05
168 h	9.10E+03	1.46E+04	9.76E+03	7.43E+05	5.29E+05	7.24E+05
192 h	1.22E+04	1.96E+04	2.90E+04	3.45E+05	3.03E+05	4.58E+05
216 h	1.67E+04	1.24E+04	3.46E+04	3.58E+05	3.06E+05	2.22E+05

Appendix 3: Fluticasone Propionate Administration

Fluticasone Propionate results

Table A13: Acquired FLP data for targeted analytes 15(S)-HETE and 5(S)-HETE using concentration. All concentrations are deemed estimates.

Analyte	**15(S)-HETE (ng/mL)**			**5(S)-HETE (ng/mL)**		
Horses	Horse 1	Horse 2	Horse 3	Horse 1	Horse 2	Horse 3
-7 Days	6.41	7.81	3.64	91.3	143	141
-6 Days	17.9	13.5	5.29	181	246	154
-5 Days	23.3	17.0	8.39	217	374	216
-4 Days	28.5	23.3	9.41	599	313	228
-3 Days	25.9	21.8	10.4	225	381	217
-2 Days	36.5	29.2	13.2	270	403	265
-1 Day	38.3	26.5	14.0	239	367	316
T0	21.2	25.4	11.8	205	330	266
2 min	28.2	23.6	11.3	196	348	279
5 min	32.6	24.0	11.4	290	305	303
10 min	20.2	22.1	10.2	208	304	233
20 min	21.2	22.4	10.9	263	294	250
30 min	29.5	17.8	12.9	201	266	288
40 min	21.7	24.5	13.6	186	353	284
50 min	27.3	21.0	10.9	245	287	258
60 min	34.7	30.5	29.2	190	387	548
90 min	37.6	30.4	11.3	341	380	267
2 h	35.9	30.6	26.6	335	419	455
2.5 h	31.6	22.7	11.6	209	432	252
3 h	60.7	29.2	14.9	580	393	294
4 h	54.2	35.7	15.3	293	516	380

6 h	55.2	28.5	14.6	474	448	371
8 h	50.7	28.2	13.2	305	413	254
12 h	21.3	18.0	12.6	196	258	230
24 h	30.1	20.4	9.32	327	257	270
48 h	29.0	21.3	8.86	179	322	204
72 h	24.6	18.5	5.87	444	247	152
96 h	31.9	22.0	8.90	196	344	215
120 h	20.2	21.8	9.02	211	357	244
144 h	26.3	20.6	12.0	274	330	218
168 h	22.9	20.3	10.9	155	161	253

Table A14: Acquired FLP data for targeted analyte 18-HEPE using concentration. All concentrations are deemed estimates.

Analyte	**18-HEPE (ng/mL)**		
Horses	Horse 1	Horse 2	Horse 3
-7 Days	1.51	2.04	0.182
-6 Days	3.15	3.58	0.674
-5 Days	4.39	4.68	1.05
-4 Days	4.80	6.25	1.32
-3 Days	5.10	6.05	1.67
-2 Days	7.49	8.04	2.52
-1 Day	7.46	7.77	2.71
T0	5.27	6.88	2.27
2 min	4.75	6.33	1.96
5 min	4.14	6.23	2.14
10 min	4.51	5.56	1.74
20 min	4.47	5.84	1.90
30 min	4.32	4.51	2.30
40 min	3.88	6.09	2.41

50 min	5.79	5.33	2.03
60 min	4.26	7.69	5.53
90 min	5.77	7.63	1.75
2 h	5.52	6.96	5.18
2.5 h	6.35	6.15	1.87
3 h	6.95	7.64	3.06
4 h	7.98	9.33	3.40
6 h	6.51	6.51	3.17
8 h	5.83	7.48	2.90
12 h	2.95	4.60	2.08
24 h	5.26	5.52	1.94
48 h	4.39	5.64	1.77
72 h	4.68	4.81	1.00
96 h	7.60	5.81	1.66
120 h	4.48	4.77	1.79
144 h	6.35	5.55	2.15
168 h	5.52	6.46	2.48

Table A15: Acquired FLP data for targeted analytes AEA and OEA using concentration. AEA concentrations are deemed estimates.

Analyte	**AEA (ng/mL)**			**OEA (ng/mL)**		
Horses	Horse 1	Horse 2	Horse 3	Horse 1	Horse 2	Horse 3
-7 Days	2.79	0.0426	0.589	ND	ND	2.65
-6 Days	2.78	ND	0.505	ND	ND	1.05
-5 Days	2.82	0.0342	0.541	ND	0.520	1.19
-4 Days	2.78	0.0736	0.507	ND	ND	1.45
-3 Days	2.82	0.0580	0.504	ND	ND	2.52
-2 Days	2.81	0.0639	0.512	0.159	0.529	3.32
-1 Day	2.82	0.0729	0.538	ND	0.109	3.86

T0	2.80	0.0665	0.557	ND	0.816	1.62
2 min	2.81	0.0907	0.538	ND	1.45	1.52
5 min	2.84	0.0834	0.546	ND	0.0631	1.12
10 min	2.82	0.0750	0.561	ND	0.193	2.00
20 min	2.82	0.0570	0.542	ND	0.542	1.61
30 min	2.81	0.100	0.530	ND	0.962	0.865
40 min	2.81	0.0820	0.557	ND	0.254	1.49
50 min	2.84	0.0789	0.561	ND	0.357	1.68
60 min	2.79	0.0393	0.535	ND	ND	1.07
90 min	2.87	0.0712	0.548	ND	ND	1.93
2 h	2.87	0.129	0.526	0.239	2.20	1.89
2.5 h	2.85	0.117	0.558	0.115	1.64	2.58
3 h	2.86	0.123	0.570	0.555	1.99	4.53
4 h	2.78	0.0794	0.552	ND	1.44	4.48
6 h	2.79	0.0665	0.505	ND	ND	1.25
8 h	2.81	0.104	0.568	0.540	2.83	3.38
12 h	2.82	0.148	0.557	0.0368	4.25	1.65
24 h	2.86	0.184	0.573	0.918	3.82	2.42
48 h	2.86	0.115	0.631	1.00	1.33	3.83
72 h	2.90	0.124	0.650	3.77	3.69	10.2
96 h	2.81	0.0906	0.600	ND	1.21	4.86
120 h	2.83	0.0636	0.572	ND	1.03	4.01
144 h	2.80	0.0589	0.530	0.688	0.339	2.44
168 h	2.82	0.131	0.590	2.61	2.33	4.64

Table A16: Acquired FLP data for targeted analytes HC and C using concentration. HC concentrations are deemed estimates.

Analyte	**HC (ng/mL)**			**C (ng/mL)**		
Horses	Horse 1	Horse 2	Horse 3	Horse 1	Horse 2	Horse 3
-7 Days	58.2	40.7	16.3	1.87	1.38	1.76
-6 Days	71.7	38.8	17.1	1.39	1.31	1.29
-5 Days	48.3	3.76	7.69	1.24	0.481	0.996
-4 Days	47.2	ND	10.8	1.25	0.207	0.998
-3 Days	34.2	ND	15.7	0.977	0.200	0.838
-2 Days	34.9	ND	14.1	0.962	0.297	1.02
-1 Day	48.3	ND	ND	1.32	0.0978	0.449
T0	74.9	ND	48.9	2.11	0.267	1.56
2 min	57.1	ND	60.3	1.67	0.184	1.74
5 min	55.2	ND	59.2	1.60	0.176	1.73
10 min	54.5	ND	73.8	1.72	0.156	2.19
20 min	52.8	ND	63.5	1.57	0.159	1.96
30 min	48.0	ND	62.3	1.52	0.121	1.91
40 min	44.3	ND	54.0	1.42	0.150	1.77
50 min	46.4	ND	48.2	1.47	0.130	1.63
60 min	45.1	ND	47.1	1.32	0.123	1.50
90 min	45.0	ND	37.9	1.49	0.0776	1.50
2 h	39.0	ND	31.3	1.36	0.131	1.39
2.5 h	34.5	ND	26.9	1.17	0.0955	1.31
3 h	39.2	ND	22.7	1.24	0.103	1.20
4 h	43.0	ND	26.9	1.21	0.0570	1.36
6 h	19.3	ND	15.1	0.762	0.0393	0.725
8 h	7.78	ND	2.35	0.635	0.0300	0.536
12 h	ND	ND	ND	0.488	0.110	0.539

24 h	15.1	ND	14.1	0.923	0.0978	1.13
48 h	53.6	ND	73.8	1.46	0.119	2.11
72 h	100	12.8	110	2.36	0.510	3.10
96 h	73.9	115	91.3	2.31	1.928	3.65
120 h	105	170	101	3.09	3.62	3.49
144 h	84.5	157	87.4	2.60	3.61	3.45
168 h	80.2	123	78.5	2.40	3.54	3.01

Lipid biomarkers during an FLP administration

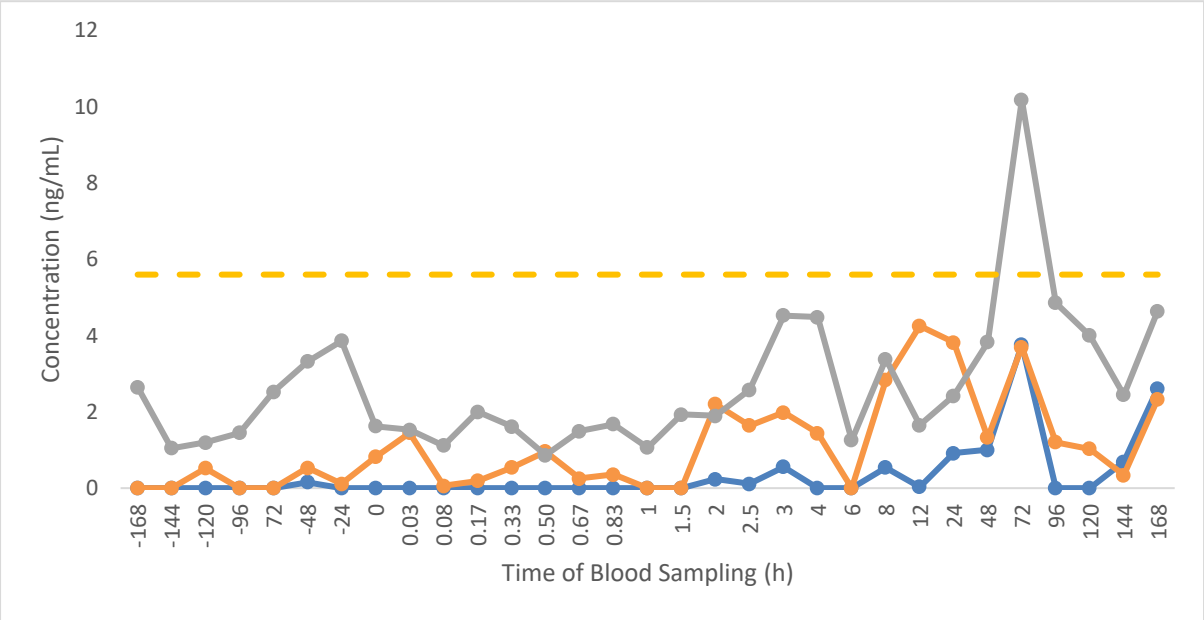

Figure A5: Effects on OEA concentrations (including pre-administration data) for 3 horses with yellow dotted line indicating the upper proposed threshold for OEA (5.6 ng/mL). (Blue indicates horse 7, orange indicates horse 8 and grey indicates horse 9)

Corticosteroid biomarkers during an FLP administration

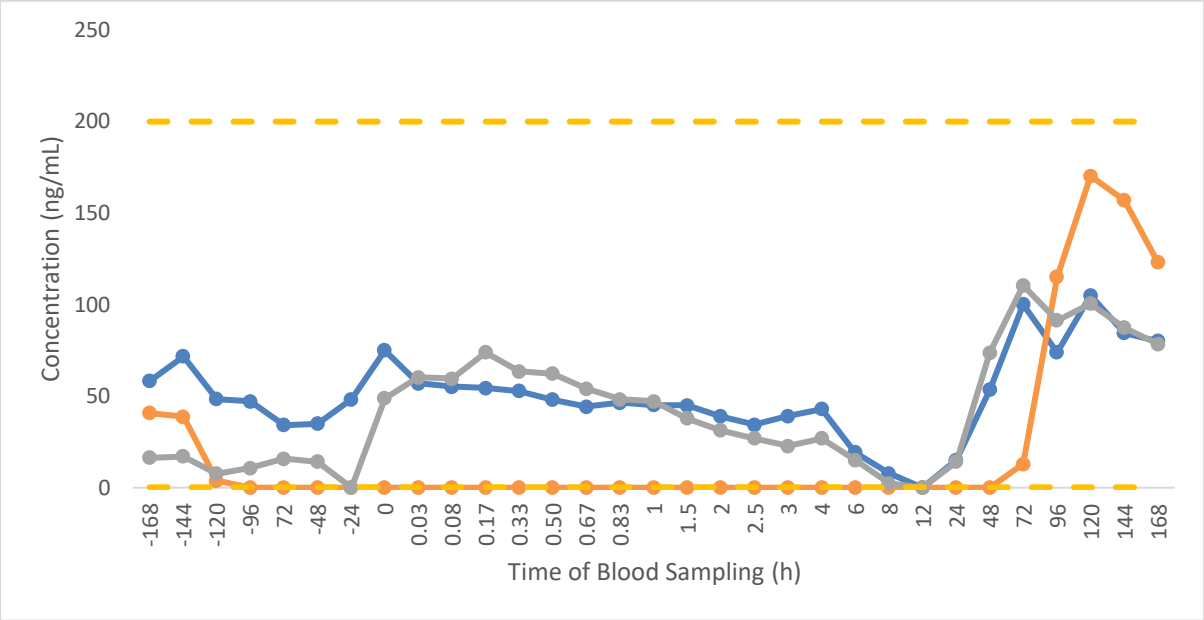

Figure A6: Effects on estimated HC for 3 horses during an FLP administration with pre-administration samples (Upper PRL: 200 ng/mL and lower PRL: 0.04 ng/mL) (blue indicates horse 7, orange indicates horse 8 and grey indicates horse 9).

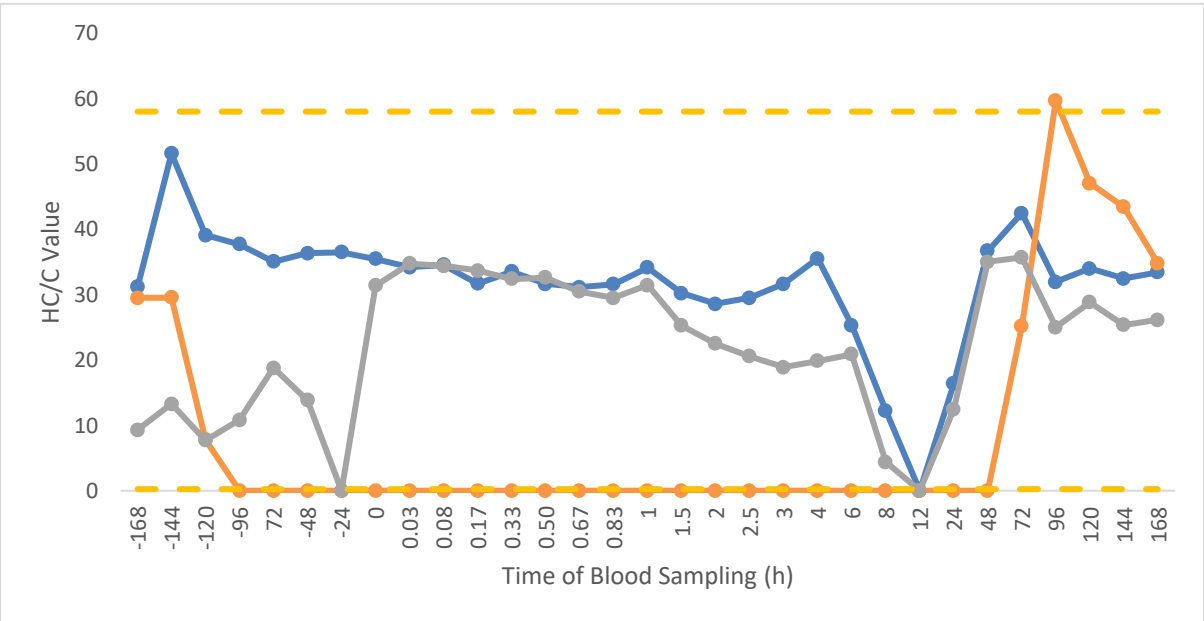

Figure A7: Effects on HC/C for 3 horses during an FLP administration with pre-administration samples (Upper PRL: 58 and lower PRL: 0.24) (blue indicates horse 7, orange indicates horse 8 and grey indicates horse 9).

Individual Reference Limits throughout an FLP administration

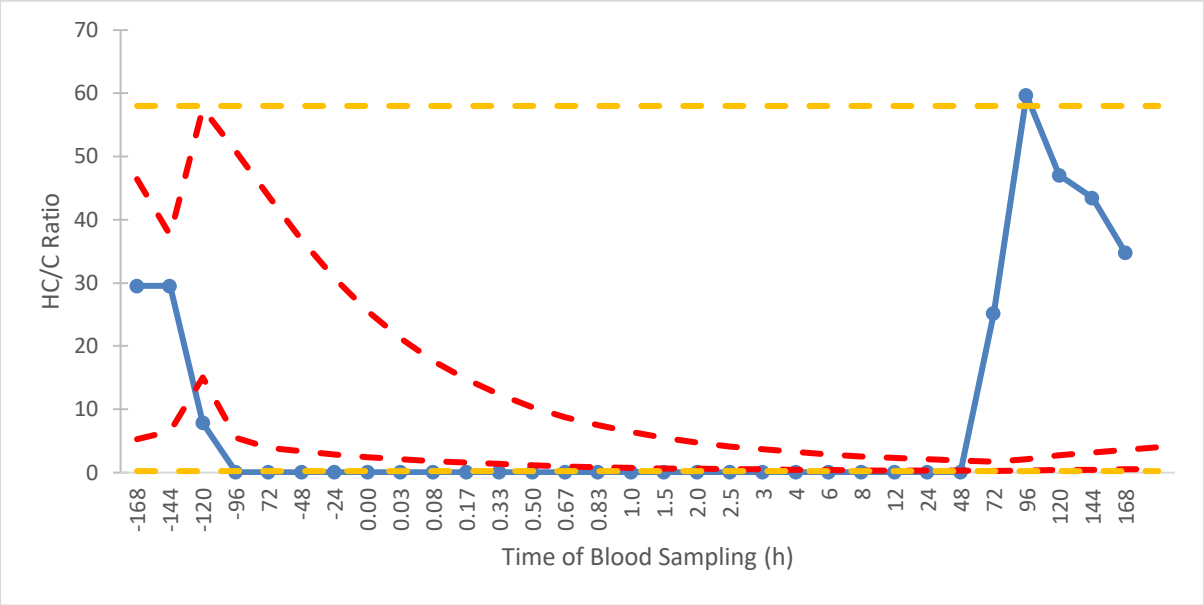

Figure A8: Intra-individual profile for the FLP administration for horse 8 using HC/C ratio (Legend: blue = HC/C ratio, red = individual upper and lower thresholds, yellow = proposed population upper (58) and lower thresholds (0.24)).

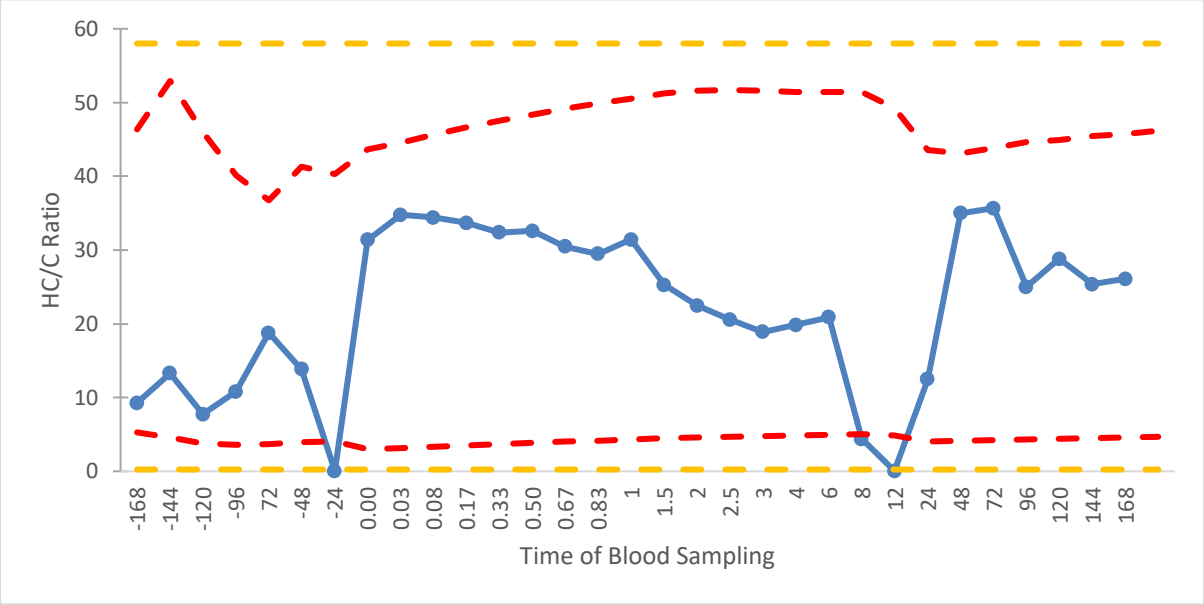

Figure A9: Intra-individual profile for the FLP administration for horse 9 using HC/C ratio (Legend: blue = HC/C ratio, red = individual upper and lower thresholds, yellow = proposed population upper (58) and lower thresholds (0.24)).

Appendix 4: Bisphosphonate administrations

Instrument parameters for bisphosphonate administrations

Table A17: MS Conditions for Bisphosphonates.

Conditions	Setting
Interface Temperature (°C)	300
DL Temperature (°C)	240
Nebulising Gas Flow (L/min)	2.30
Heating Gas Flow (L/min)	5.00
Heat Block (°C)	400
Drying Gas Flow (L/min)	5.00

Method validation preparation parameters

Linearity

Table A18: Plasma (1 mL) spike calibration preparation summary for both ZA and TA.

Spike Concentration (ng/mL)	Analyte Solution (TA and ZA)	Amount (µL)
0	-	-
0.5	Working 20 ng/mL	25
1	Working 20 ng/mL	50
2	Working 20 ng/mL	100
5	Working 200 ng/mL	25
10	Working 200 ng/mL	50
20	Working 200 ng/mL	100
50	Working 2000 ng/mL	25
100	Working 2000 ng/mL	50

200	Working 2000 ng/mL	100
500	Working 10,000 ng/mL	50
1000	Working 10,000 ng/mL	100

Sensitivity

Table A19: Plasma spike for LOD and LOQ preparation in 1 mL of equine plasma for ZA.

Spike Concentration (ng/mL)	Solution	Amount (µL)
5	Working 200 ng/mL	25
10	Working 200 ng/mL	50
20	Working 200 ng/mL	100

Table A20: Plasma spike for LOD and LOQ preparation in 1 mL of equine plasma for TA.

Spike Concentration (ng/mL)	Solution	Amount (µL)
0.5	Working 10 ng/mL	50
0.4	Working 10 ng/mL	40
0.3	Working 10 ng/mL	30
0.2	Working 10 ng/mL	20
0.1	Working 5 ng/mL	20
0.05	Working 5 ng/mL	10

Data analysis parameters for bisphosphonate administration

Table A21: Parameters for LabSolutions Insight acquisition data for LC-MS/MS 8050.

Condition	Optimised Condition
Peak Integration Conditions	
Smoothing Method	Standard
Smoothing Width	1 second
Identification Conditions	

Identification Method	Absolute
Peak Selection	Closest Peak
Window for Target Peak	5.00%
Window for Reference Peak	5.00%
Processing Time	± 0.7 min
Quantitative Conditions	
Method	Internal Standard
Calculated by	Area
Number of calibration levels	10
Type	Linear
Zero	Not Forced
Units	ng/mL

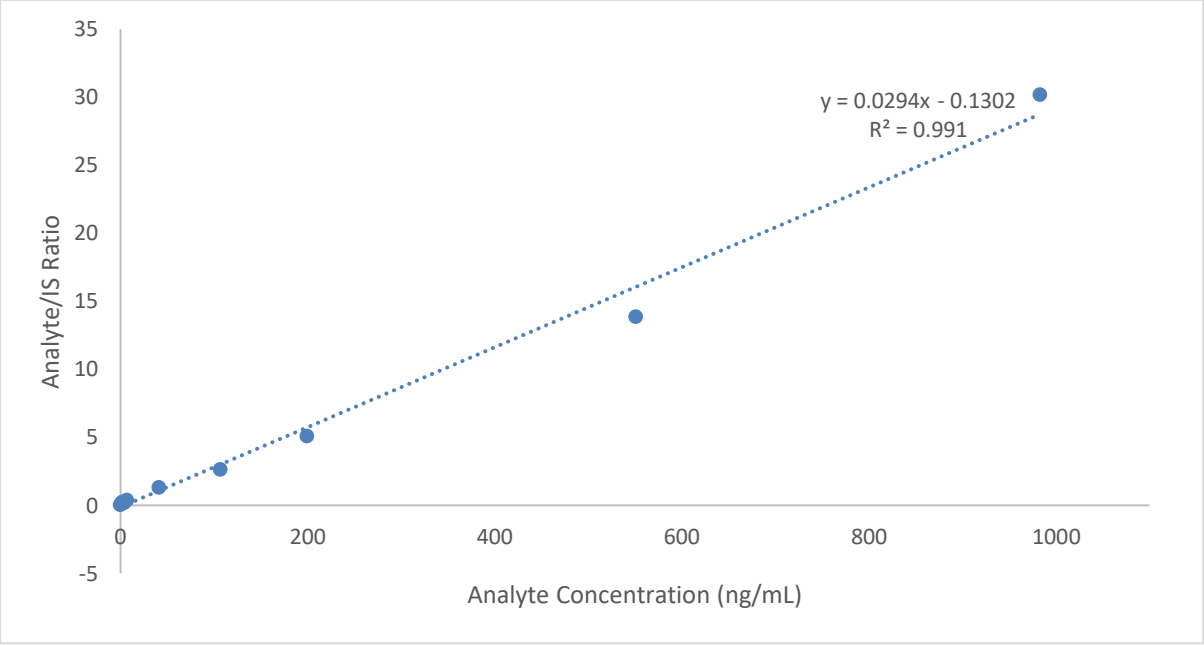

Figure A10: Linearity of ZA in equine plasma over 4 separate batches.

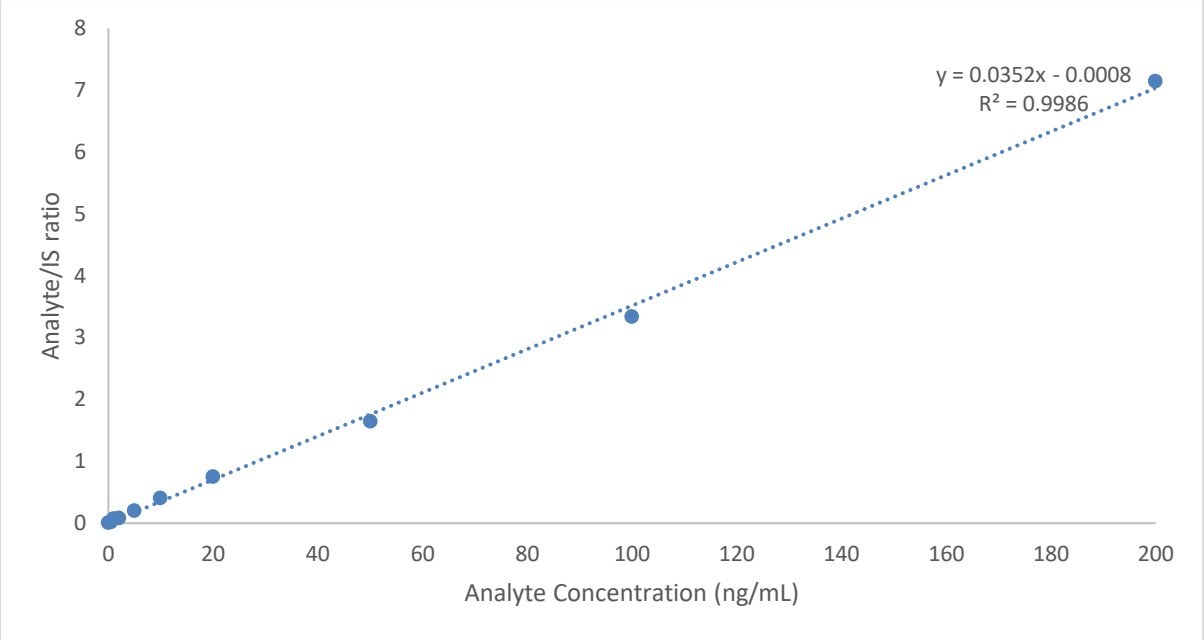

Figure A11: Linearity of TA in equine plasma over 4 separate batches up to 200 ng/mL.

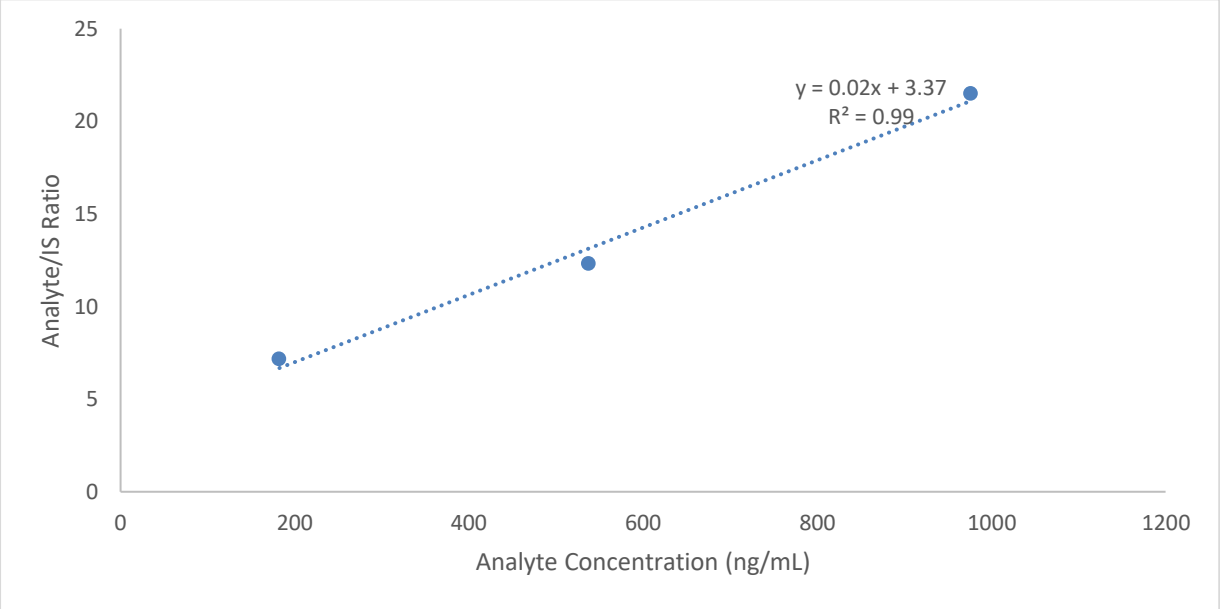

Figure A12: Linearity of TA in equine plasma over 4 separate batches between 200 - 1000 ng/mL

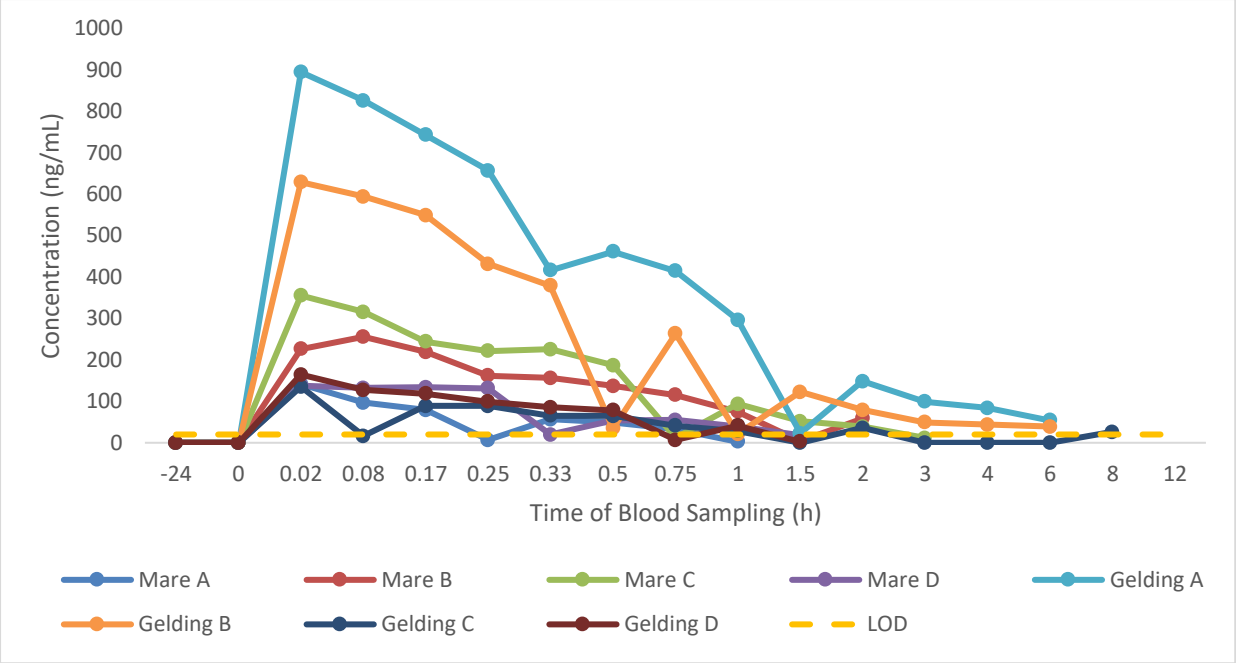

Figure A13: Elimination of ZA in 8 horses including pre-administration samples. Limit of Quantification (LOQ) of 20 ng/mL is denoted by the yellow dotted line.

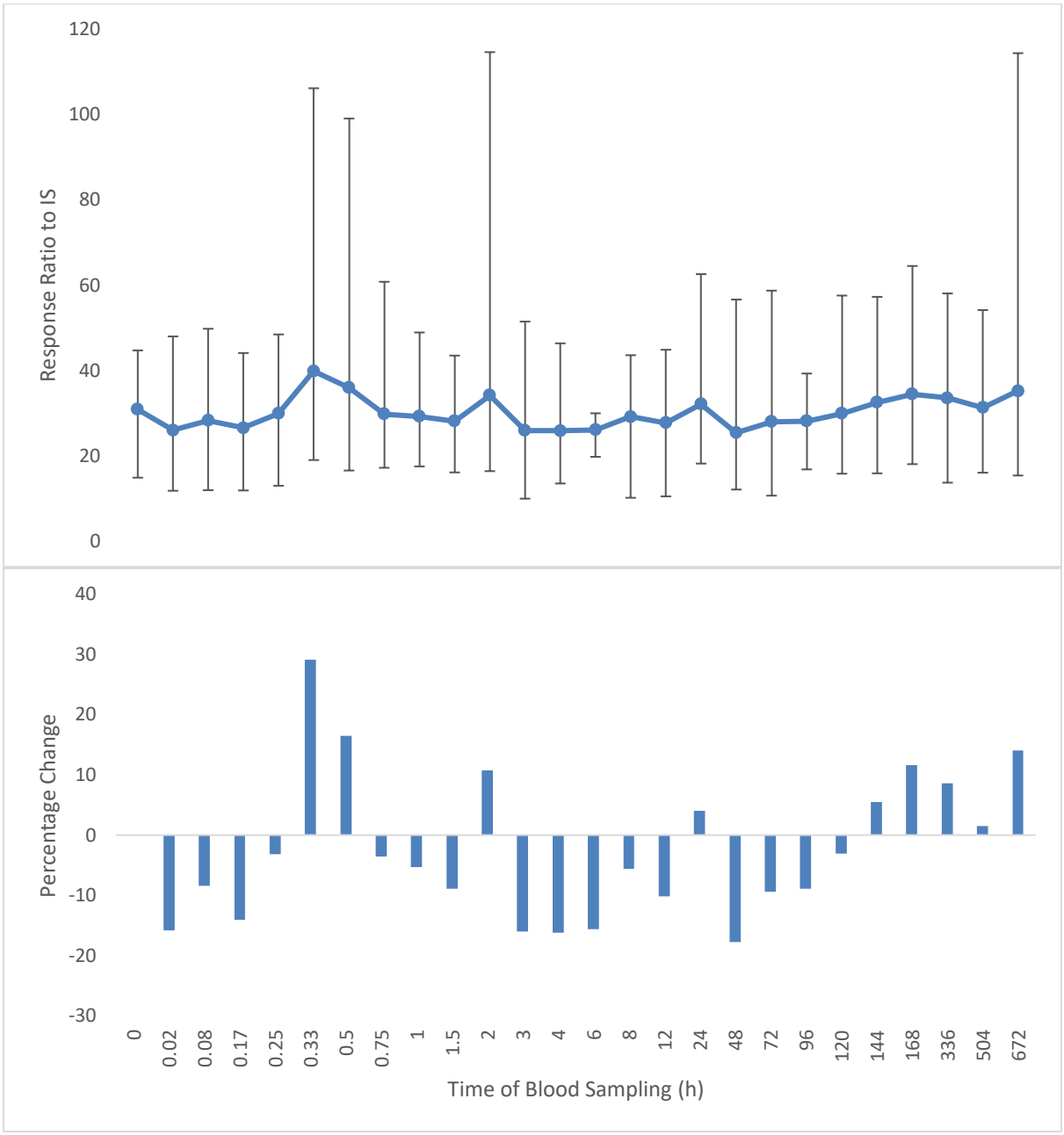

Figure A14: 12-HETE profile following a ZA administration. Top panel showing average integrated peak area with vertical bars representing the range, bottom panel showing the average percentage change (n=8)

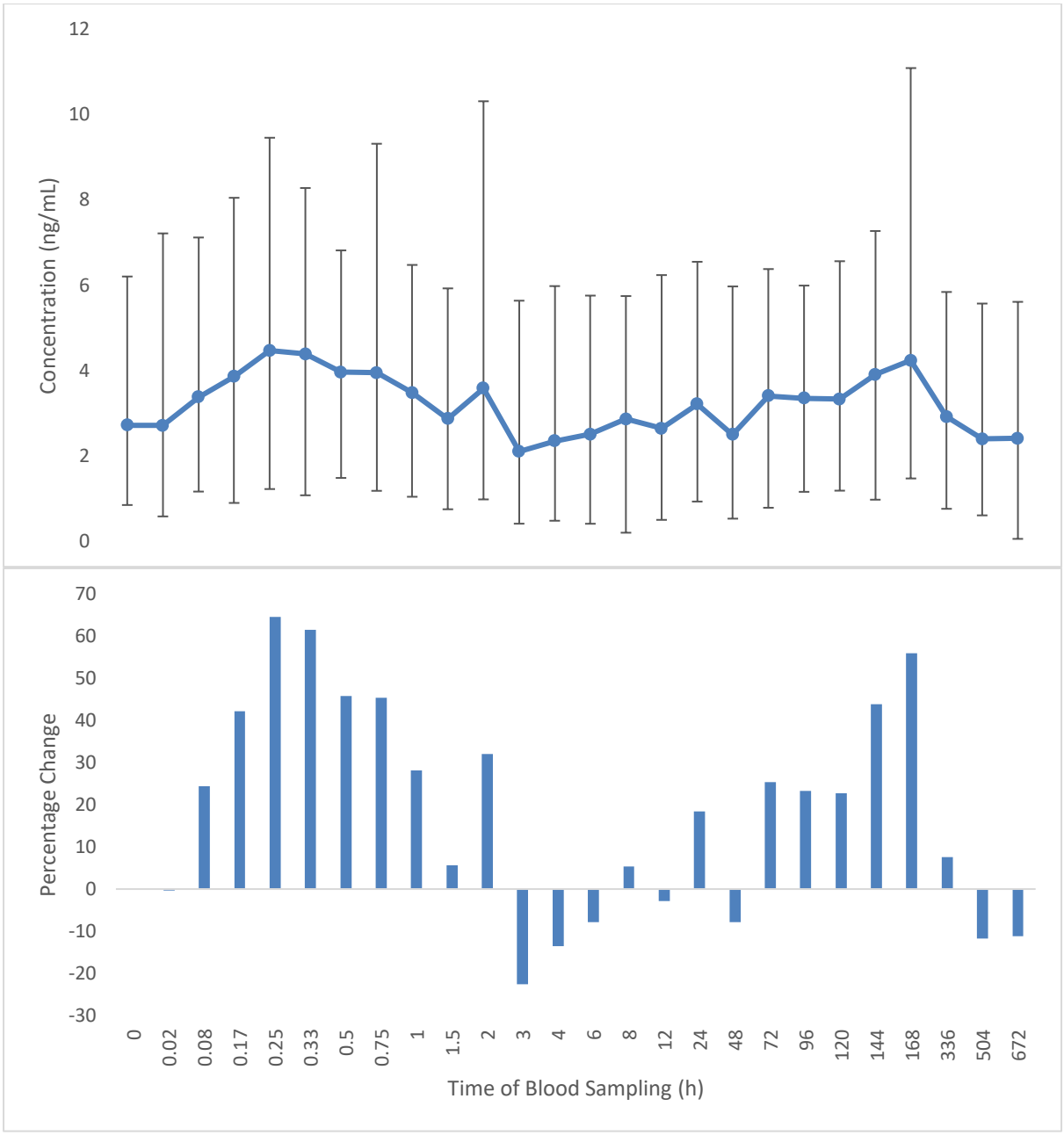

Figure A15: 13-HDHA or 17-HDHA profile following a ZA administration. Top panel showing estimated concentration with vertical bars representing the range, bottom panel showing the average percentage change (n=8)

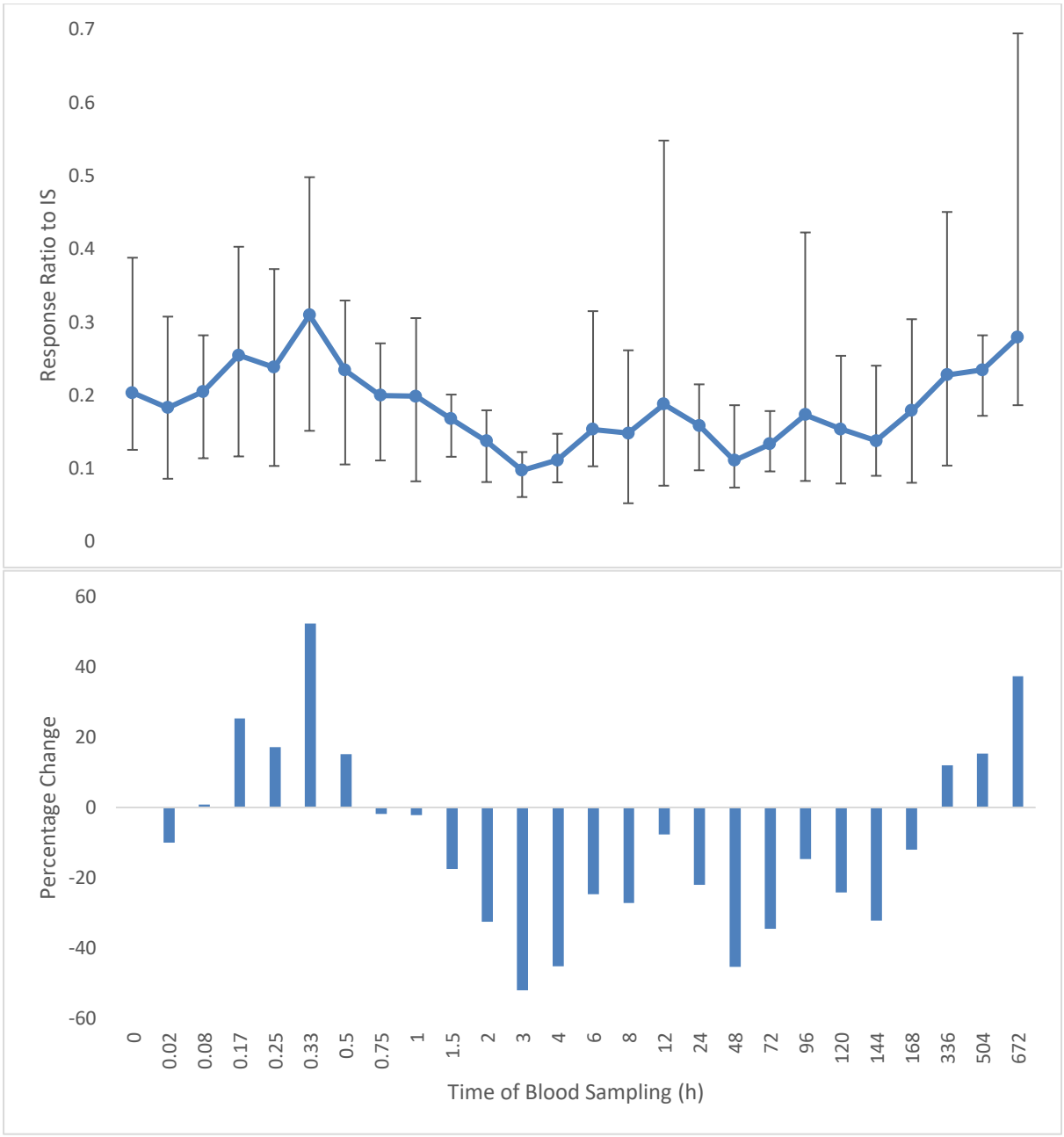

Figure A16: 15-HEDE profile following a ZA administration. Top panel showing integrated peak area with vertical bars representing the range, bottom panel showing the average percentage change (n=8)

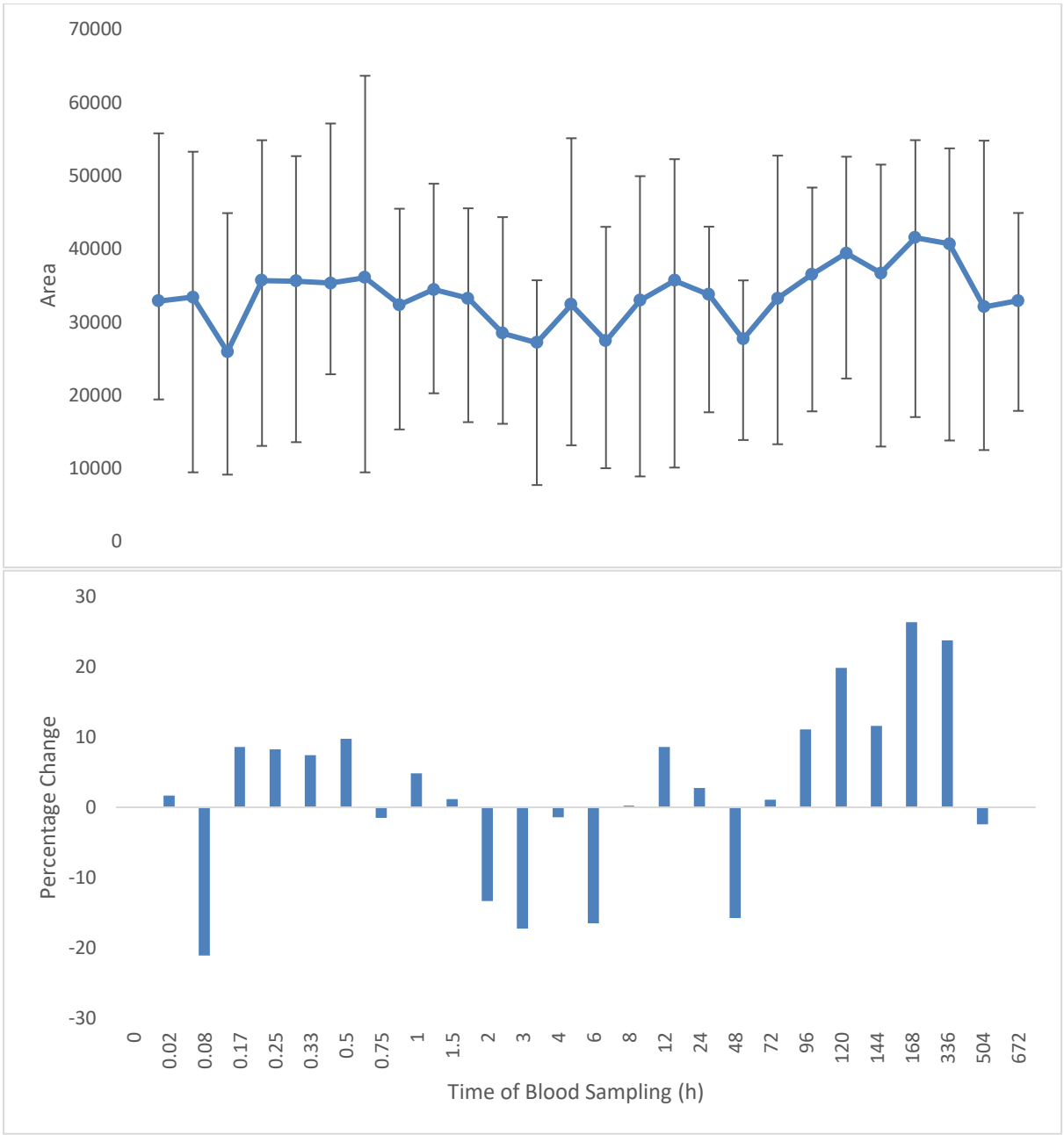

Figure A17: 15-HETE profile following a ZA administration. Top panel showing peak area and vertical bars representing the range, bottom panel showing the average percentage change (n=8)

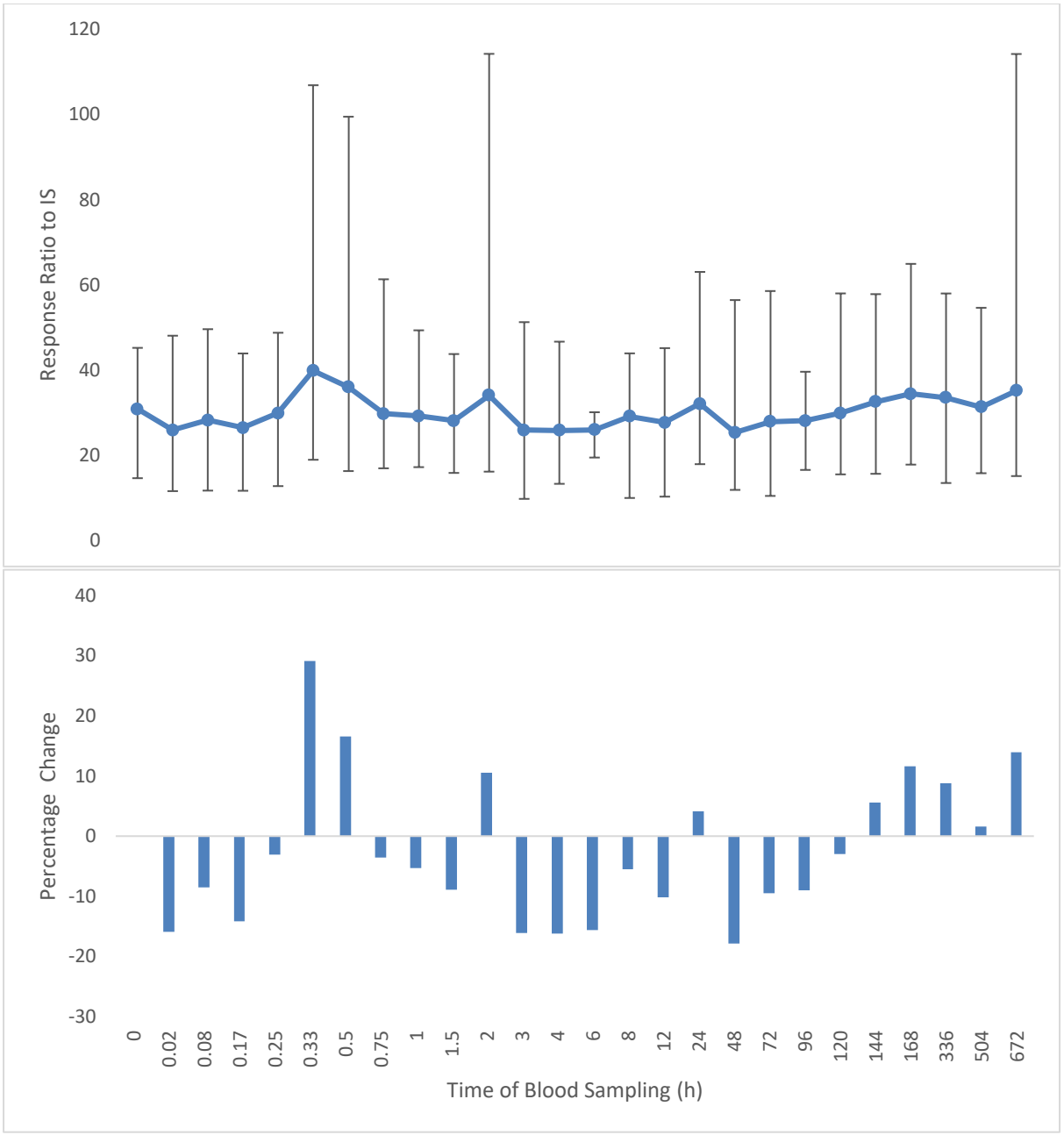

Figure A18: 5-HETE profile following a ZA administration. Top panel showing integrated peak area with vertical bars representing the range, bottom panel showing the average percentage change (n=8)

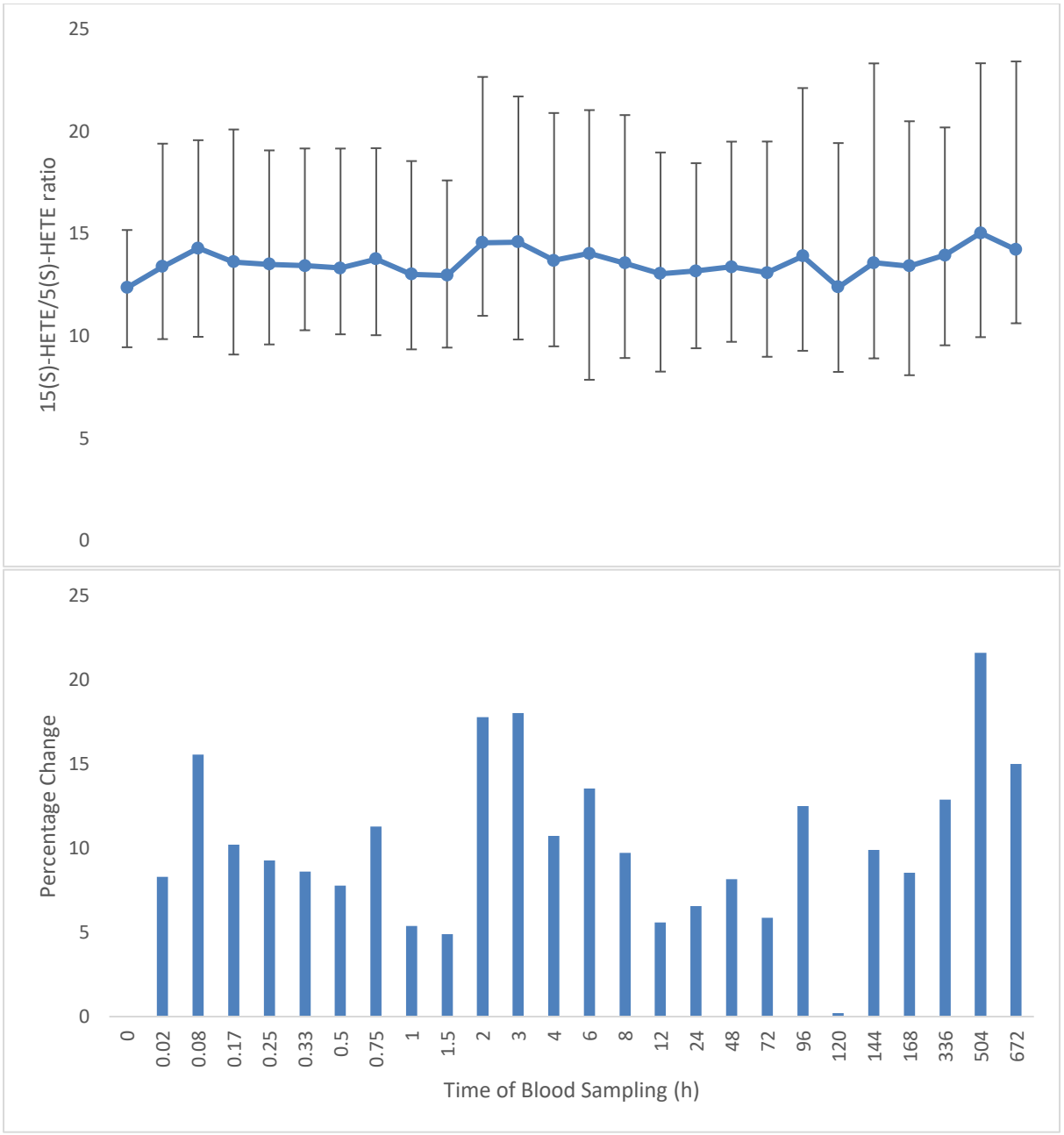

Figure A19: 5(S)-HETE/15(S)-HETE ratio profile following a ZA administration. Top panel showing the ratio with the vertical bars representing the range, bottom panel showing the average percentage change (n=8)

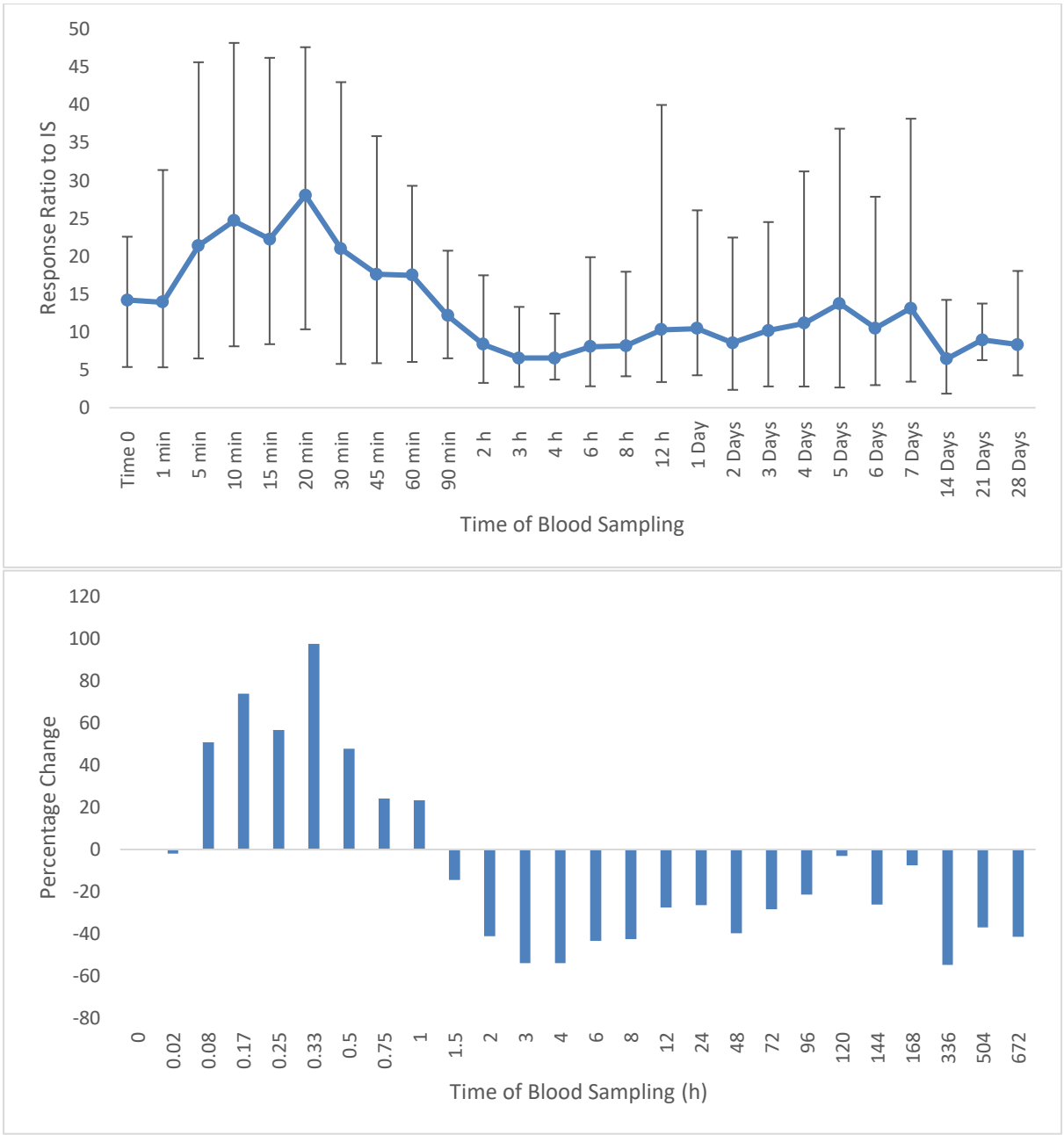

Figure A20: 9-HOTrE profile following a ZA administration. Top panel showing the integrated peak area and the vertical bars representing the range, bottom panel showing the average percentage change (n=8)

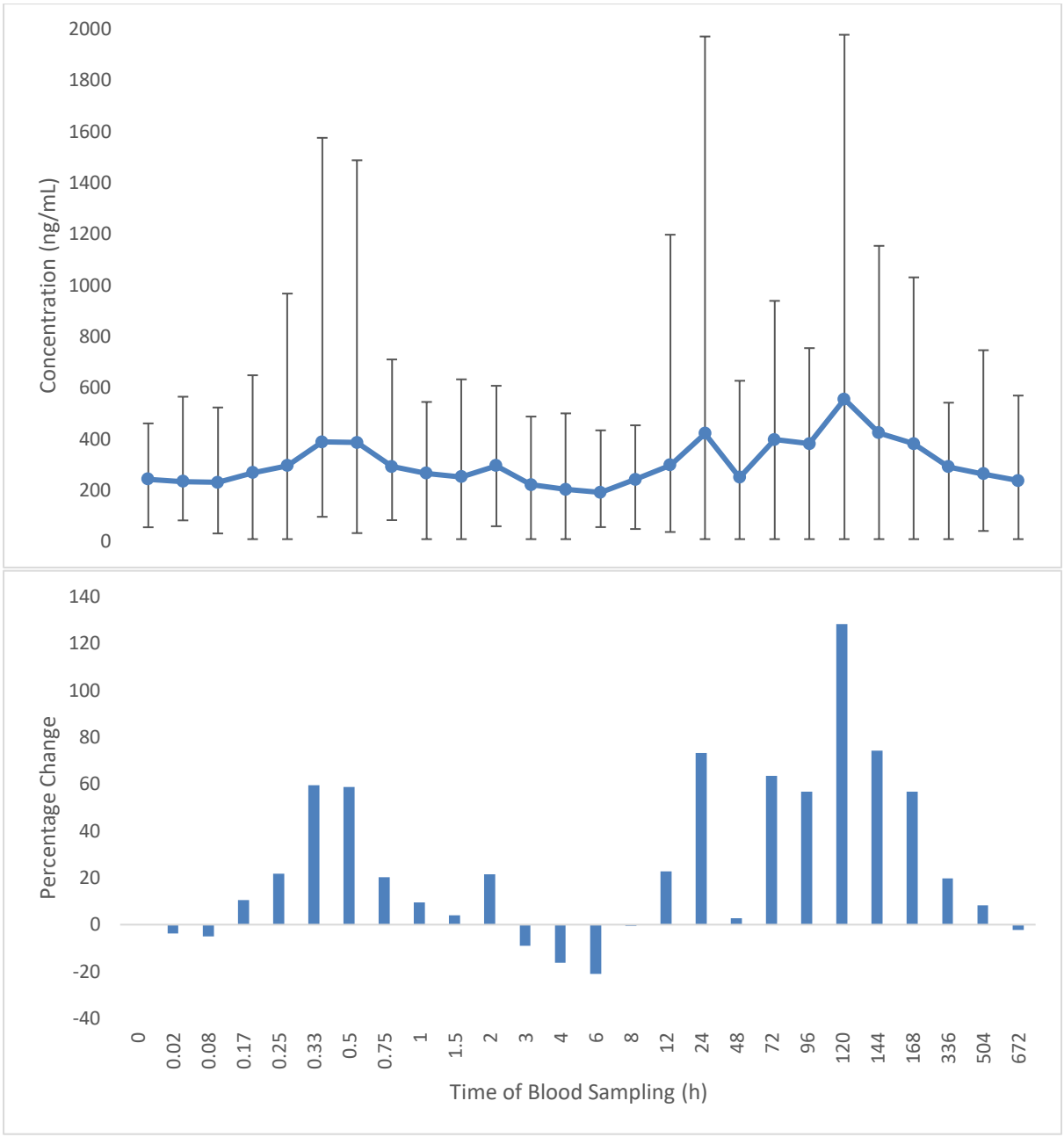

Figure A21: AA profile following a ZA administration. Top panel showing the estimated concentration and vertical bars representing the range, bottom panel showing the average percentage change (n=8)

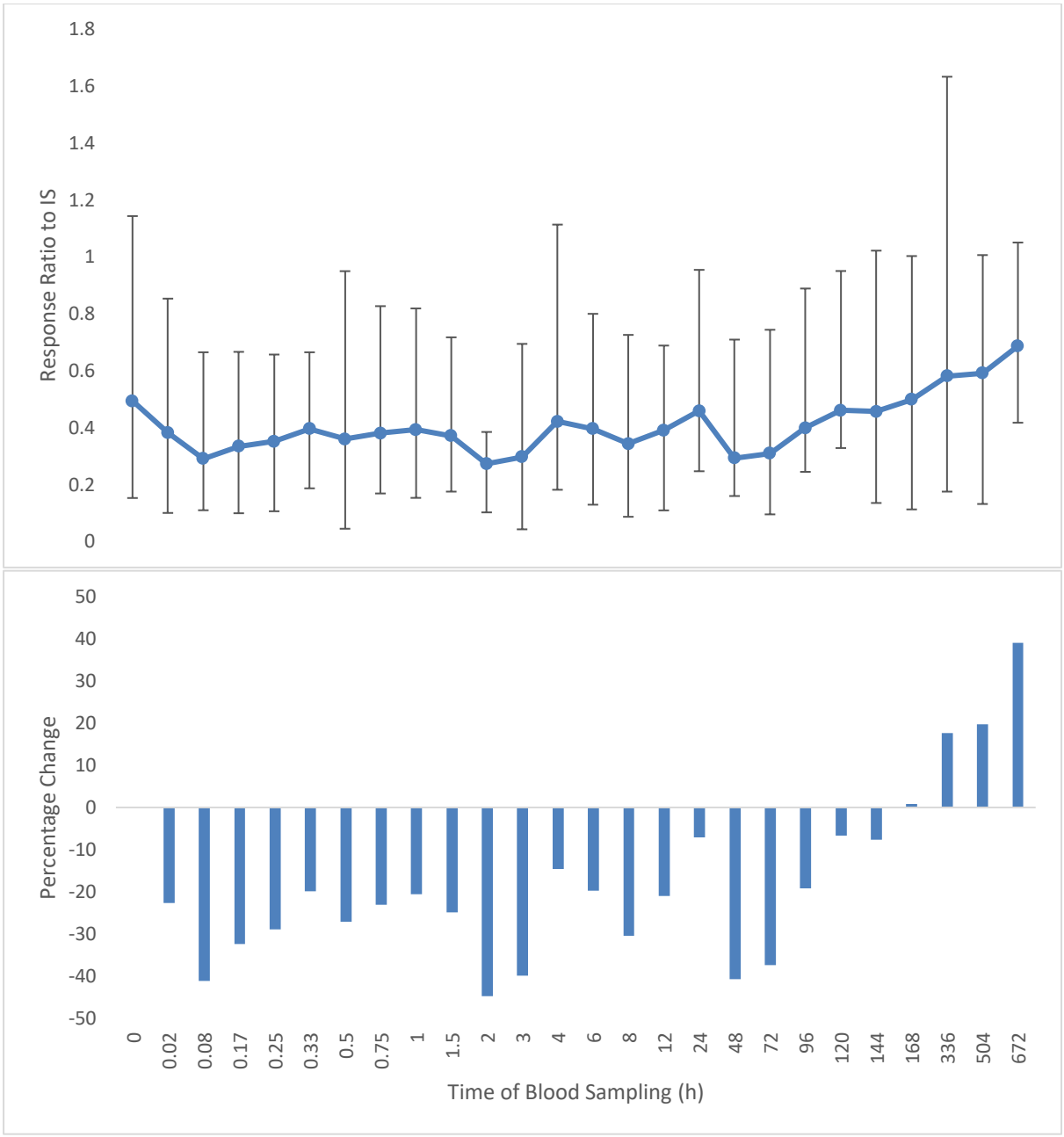

Figure A22: PGD_2 profile following a ZA administration. Top panel showing the integrated peak area with vertical bars representing the range, bottom panel showing the average percentage change (n=8)

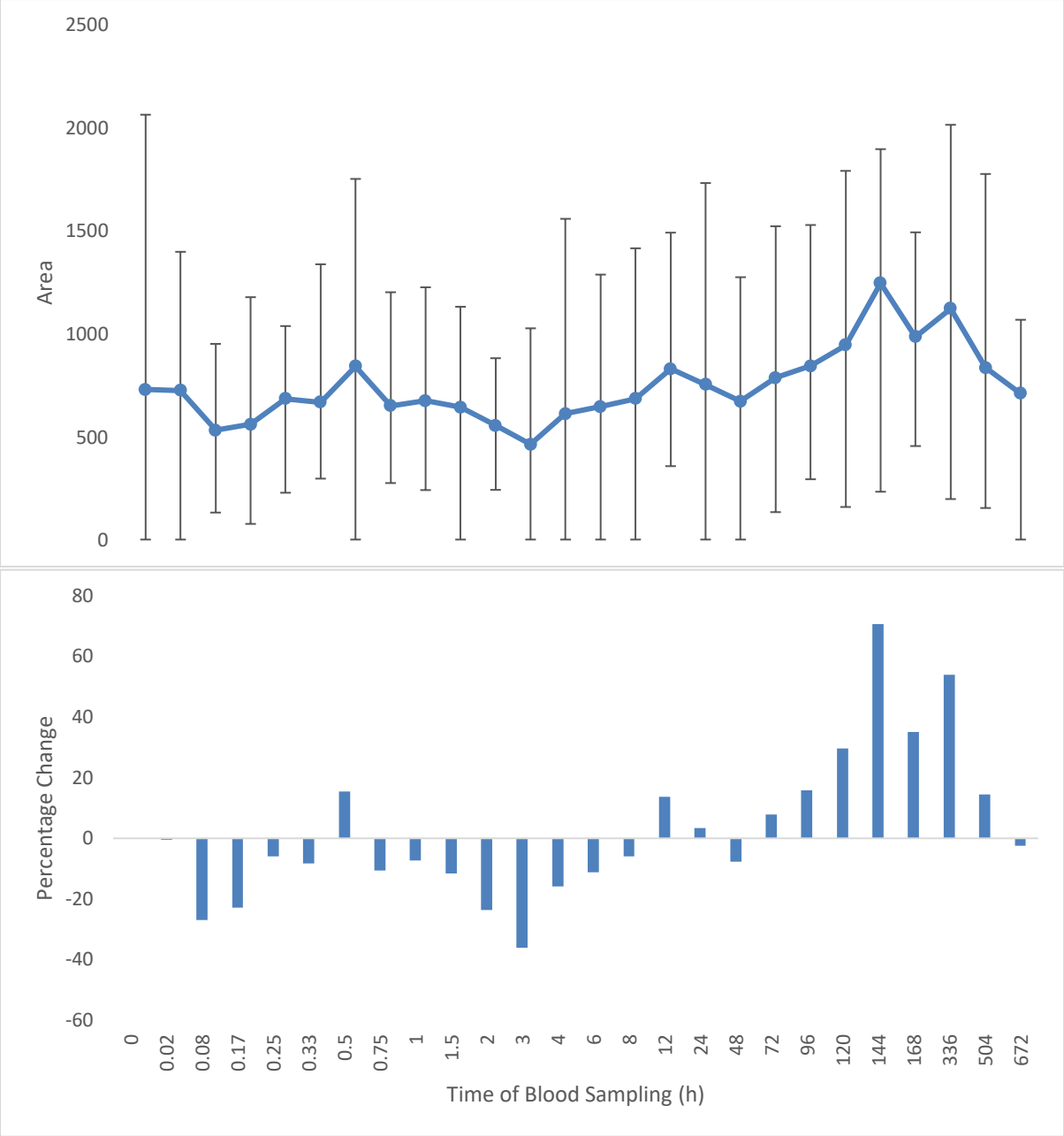

Figure A23: $PGF_{2\alpha}$ profile following a ZA administration. Top panel showing the peak area and the vertical bars representing the range, bottom panel showing the average percentage change (n=8)

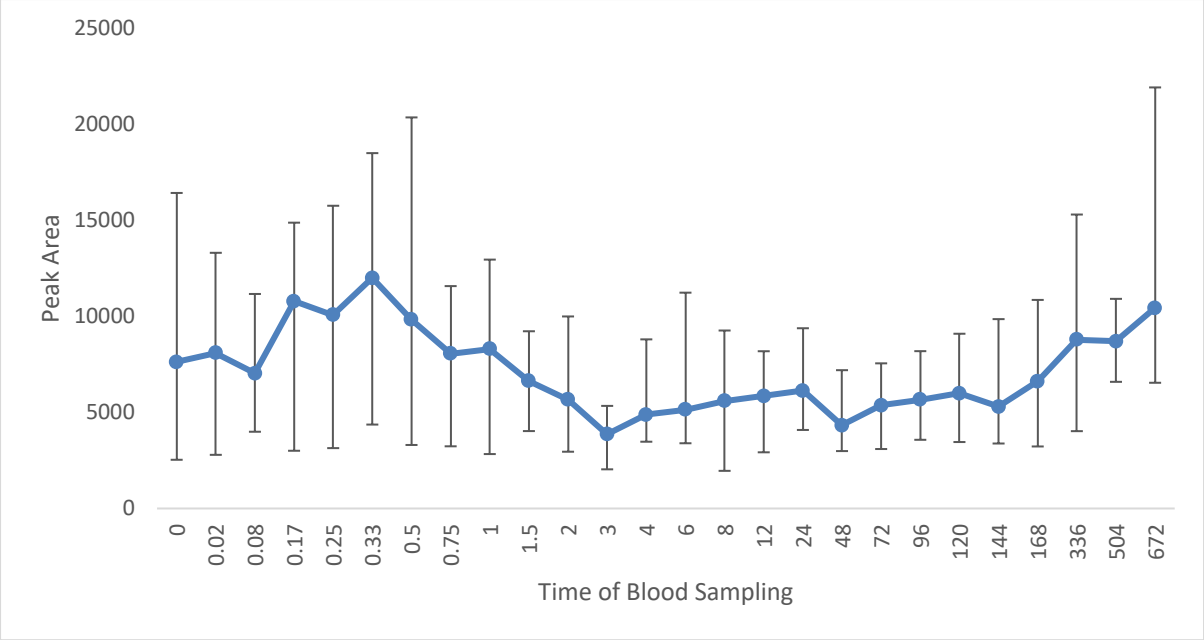

Figure A24: 11-HEDE profile following a ZA administration using peak area. Vertical bars represent range.

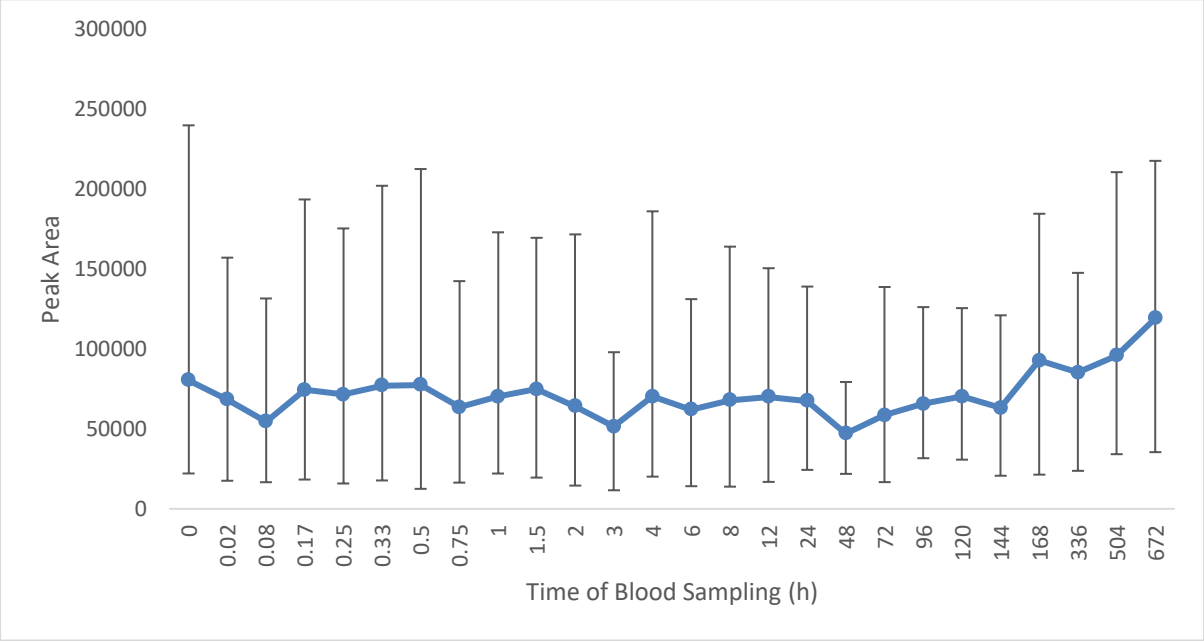

Figure A25: 14,15-DiHETE profile following a ZA administration using peak area. Vertical bars represent range.

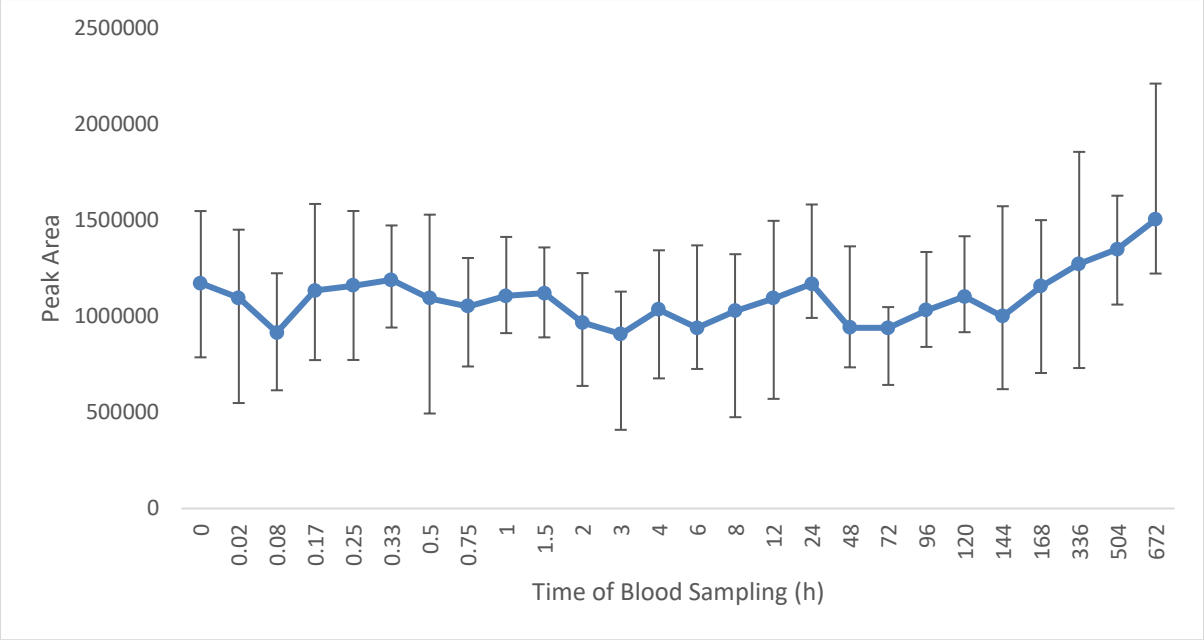

Figure A26: 9-HODE profile following a ZA administration using peak area. Vertical bars represent range.

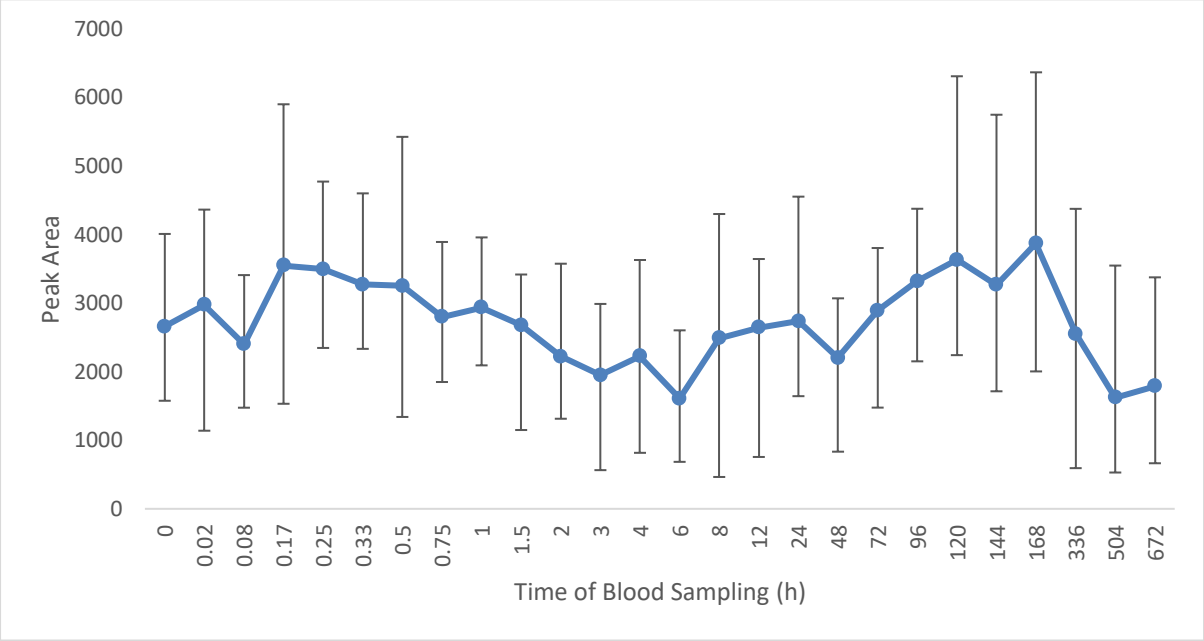

Figure A27: 20-HDHA profile following a ZA administration using peak area. Vertical bars represent range.

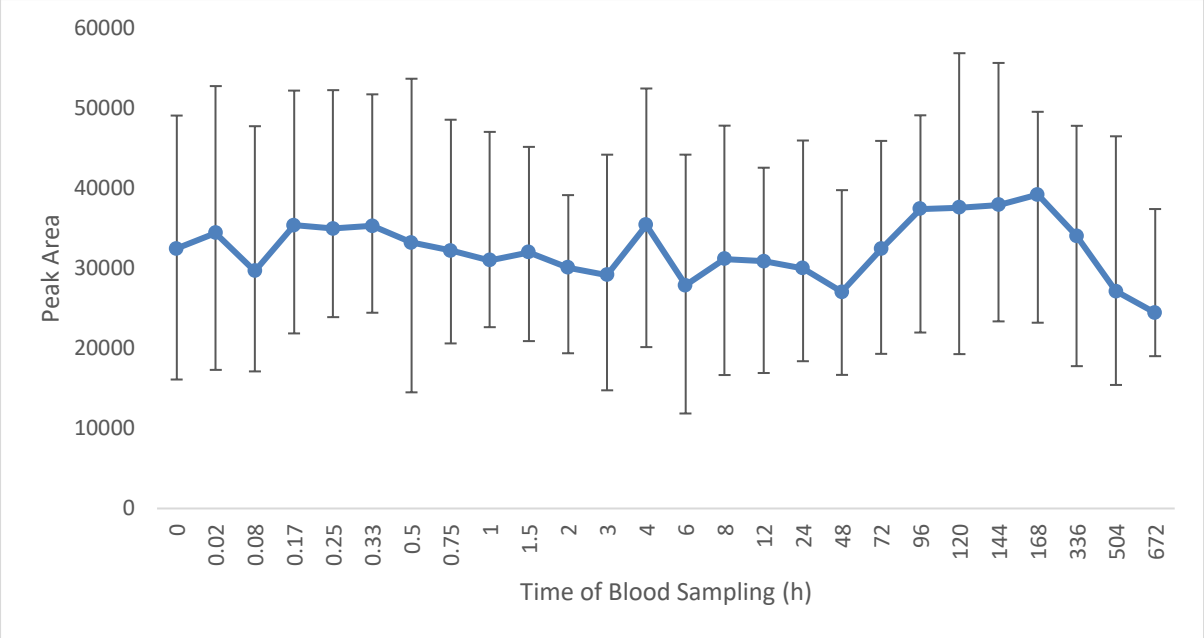

Figure A28: 4-HDHA profile following a ZA administration using peak area. Vertical bars represent range.

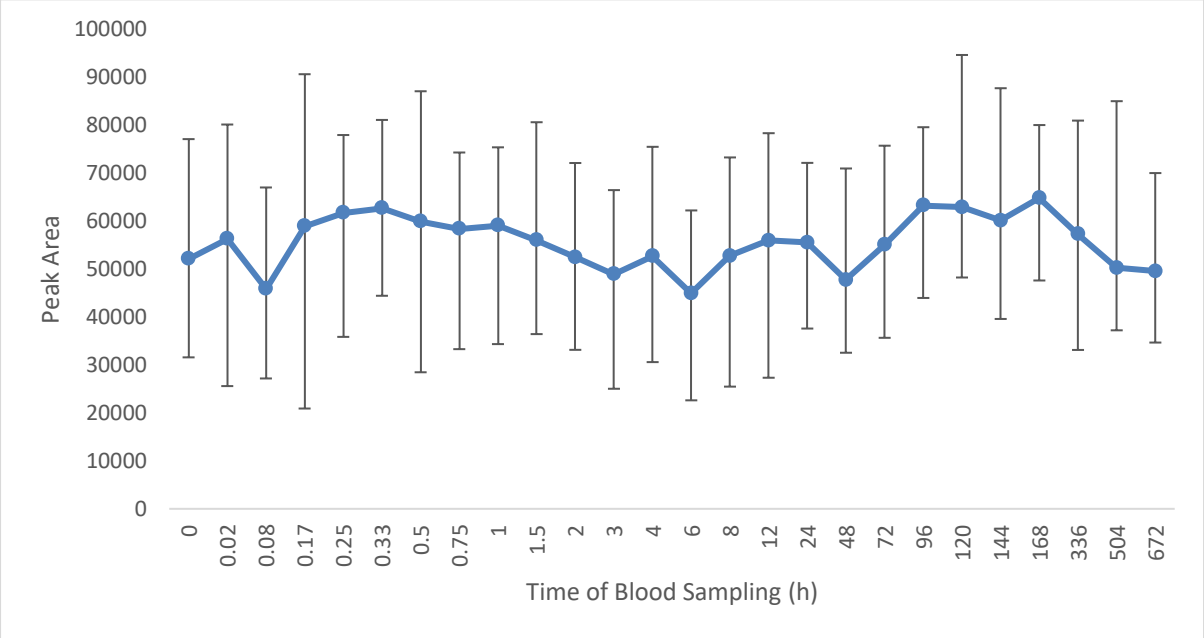

Figure A29: 9-HEPE profile following a ZA administration using peak area. Vertical bars represent range.

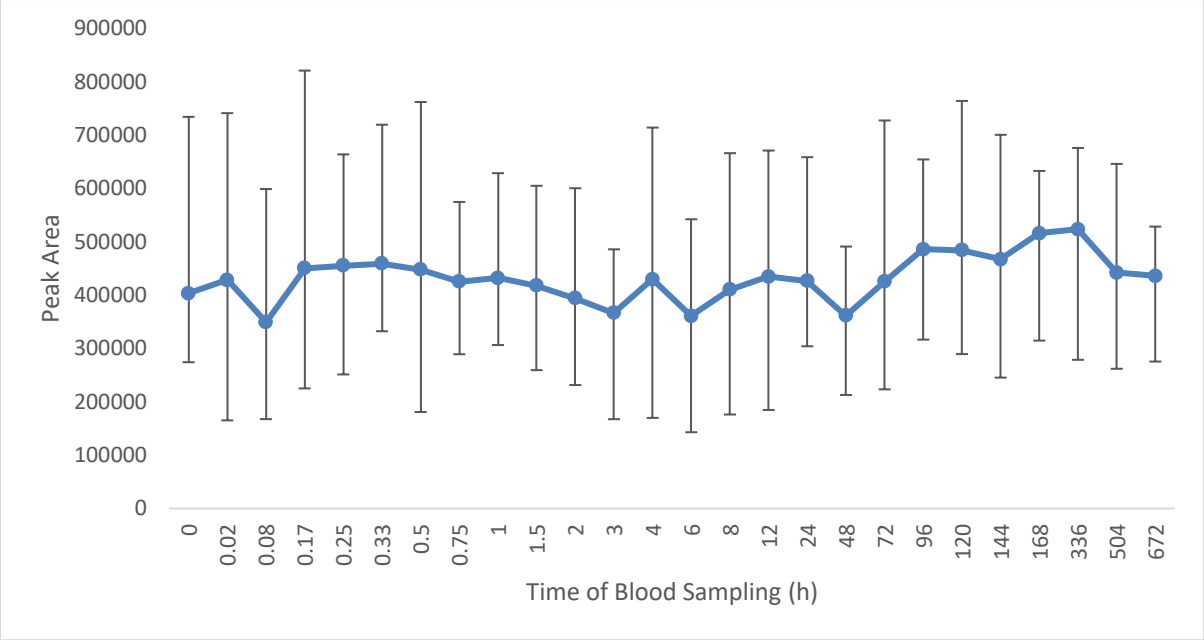

Figure A30: 9-HETE profile following a ZA administration using peak area. Vertical bars represent range.

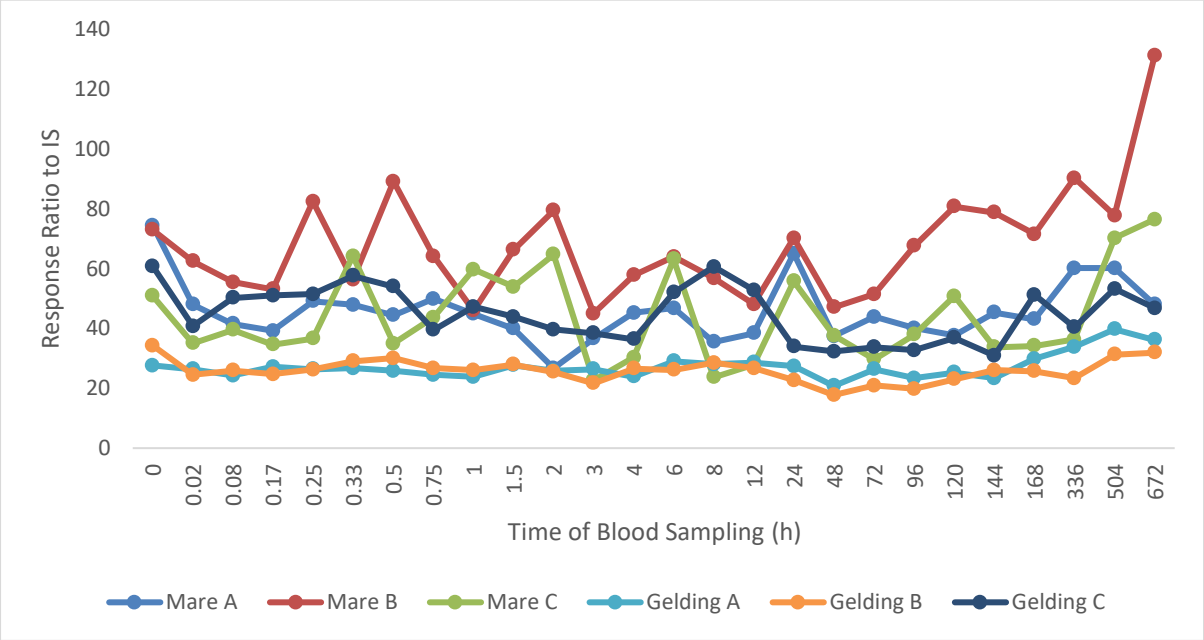

Figure A31: 13-HODE profile following a ZA administration using the area ratio between the peak area and a representative internal standard (excluding Mare 10 and Gelding 8)

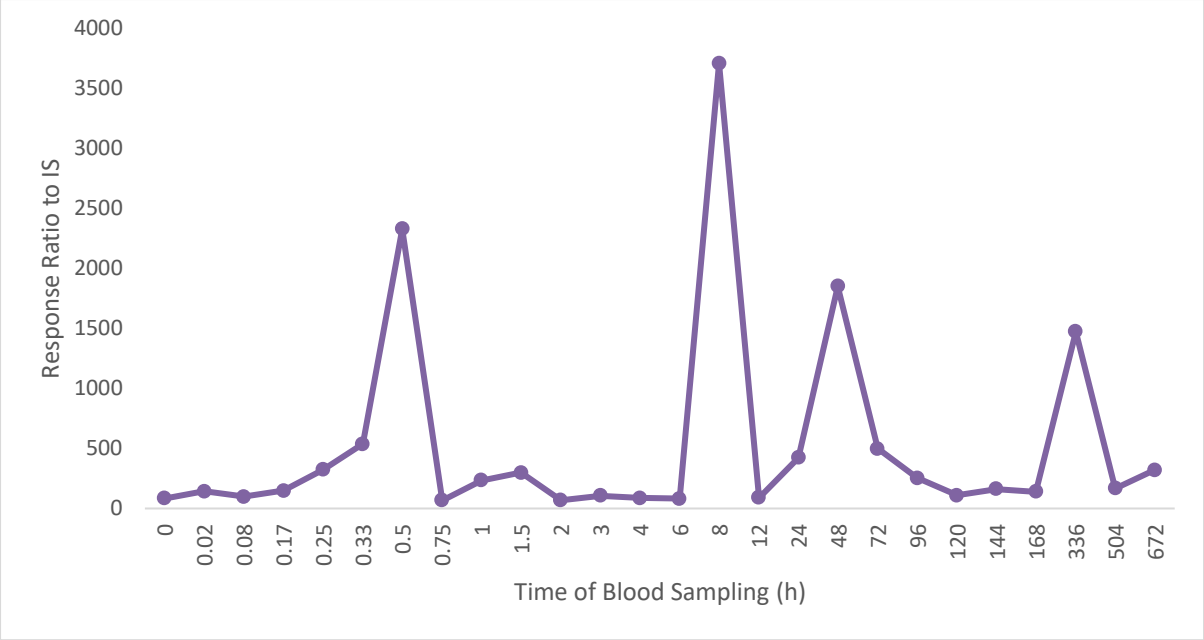

Figure A32: 13-HODE profile for Mare D following a ZA administration using the area ratio between the peak area and representative internal standard.

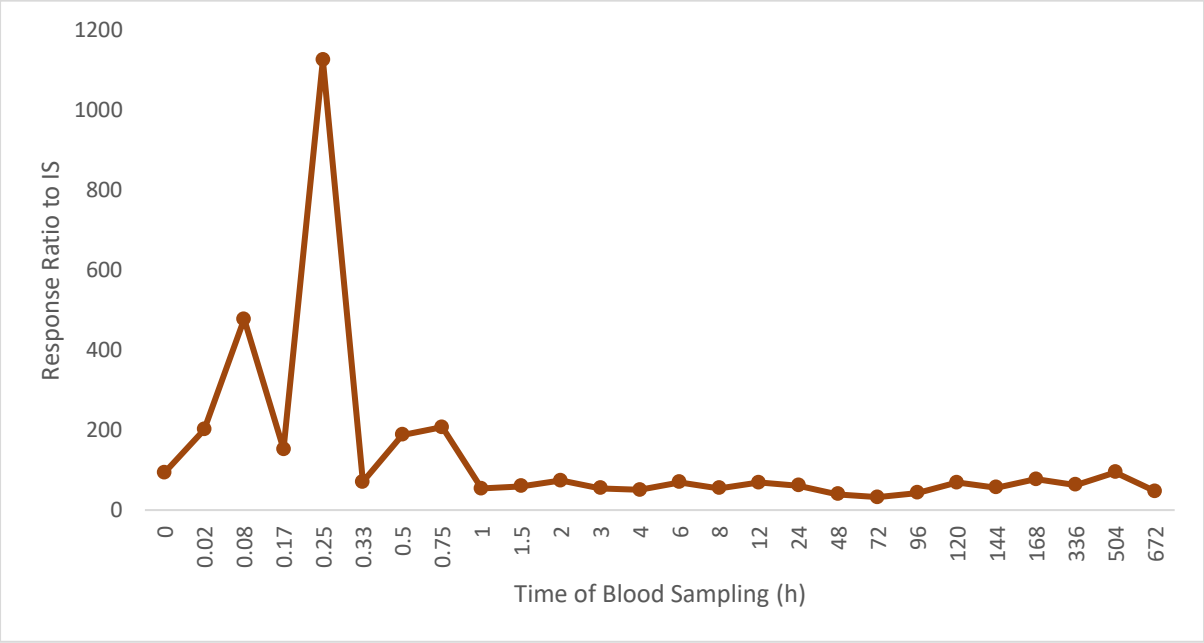

Figure A33: 13-HODE profile for Gelding D following a ZA administration using the area ratio between the peak area and representative internal standard.

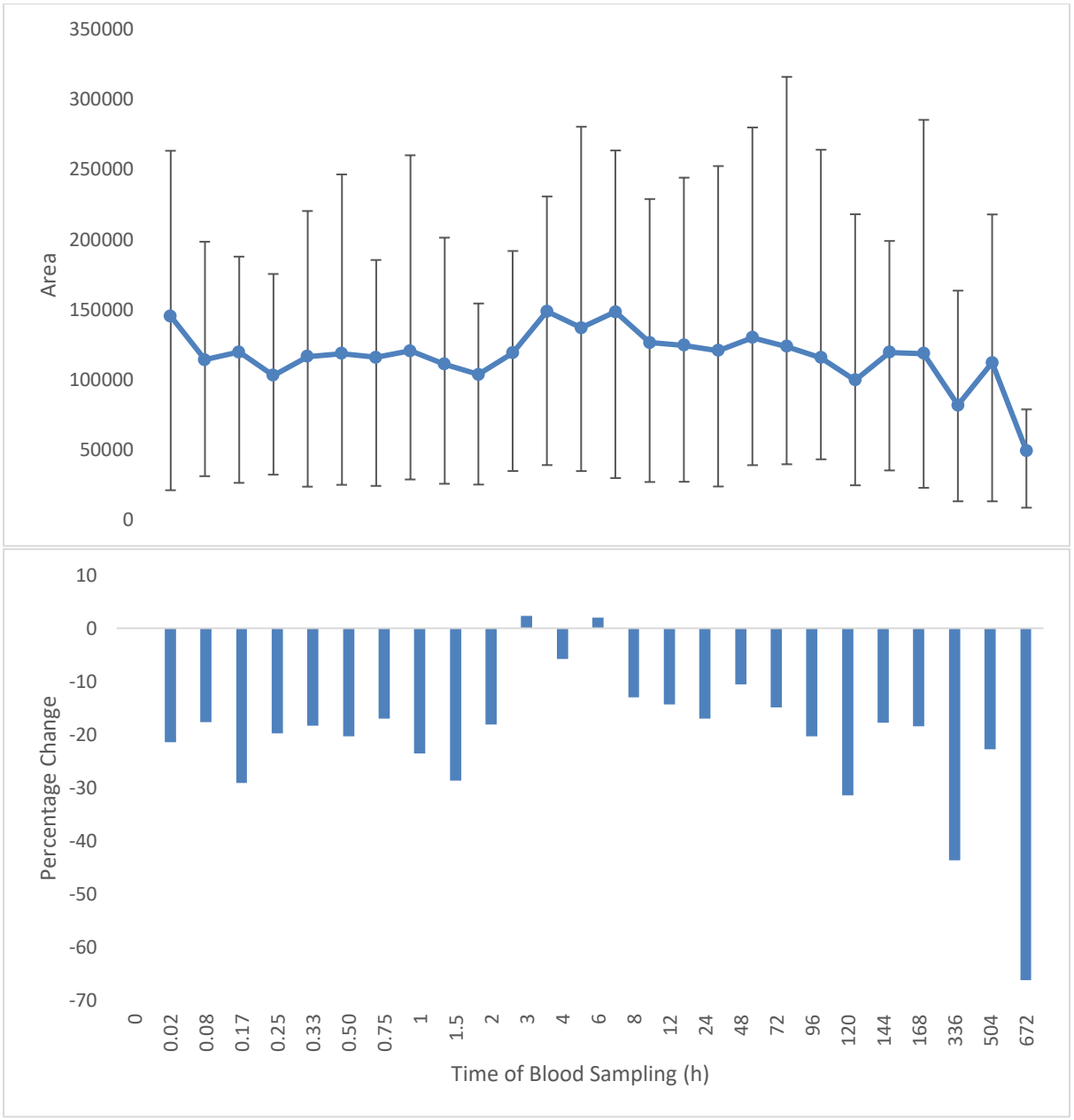

Figure A34: 12-HETE profile following a TA administration. Top panel showing peak area with vertical bars representing the range, bottom panel showing the average percentage change (n=8)

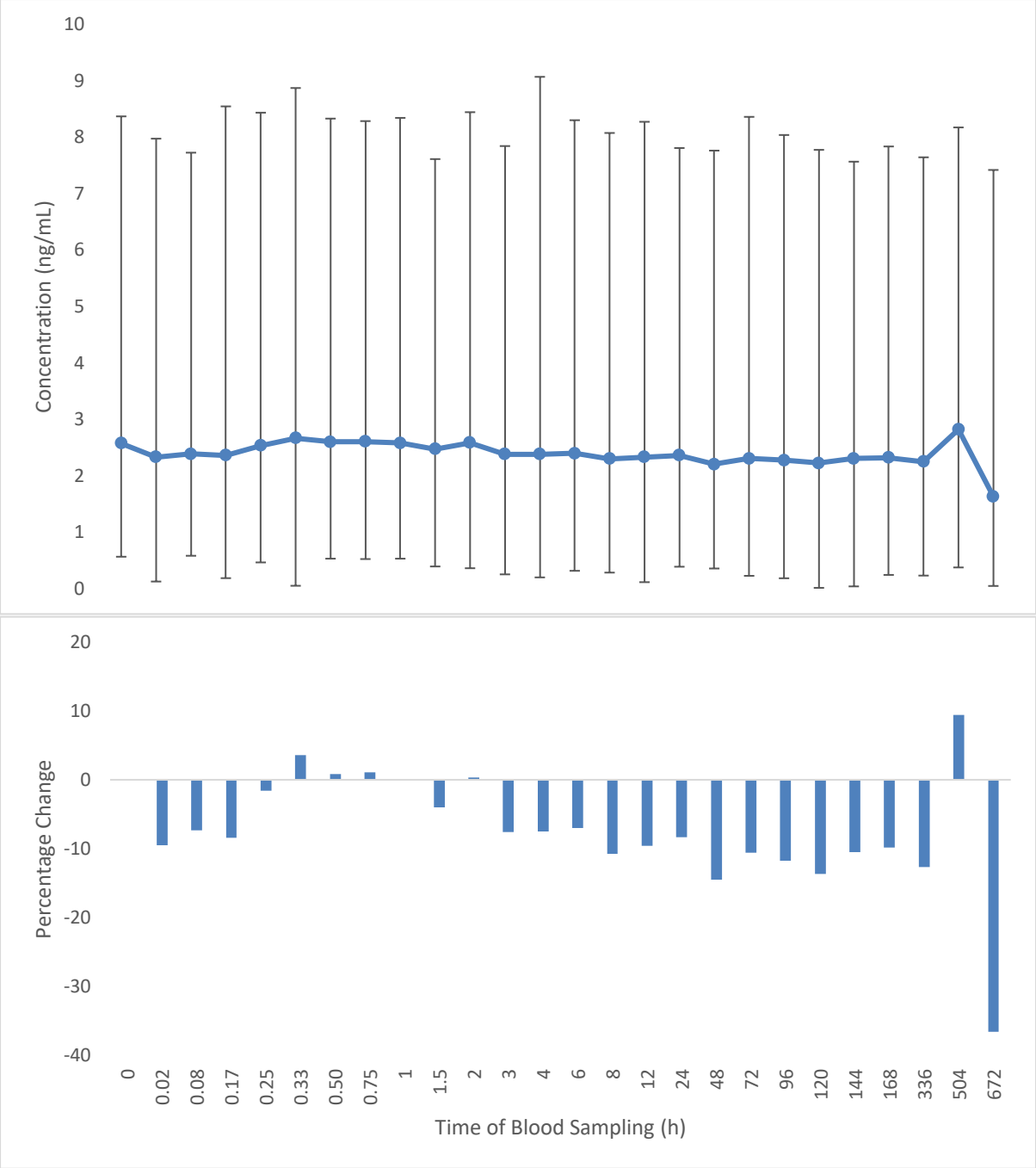

Figure A35: 13-HDHA or 17-HDHA profile following a TA administration. Top panel showing average estimated concentration with vertical bars representing the range, bottom panel showing the average percentage change (n=8)

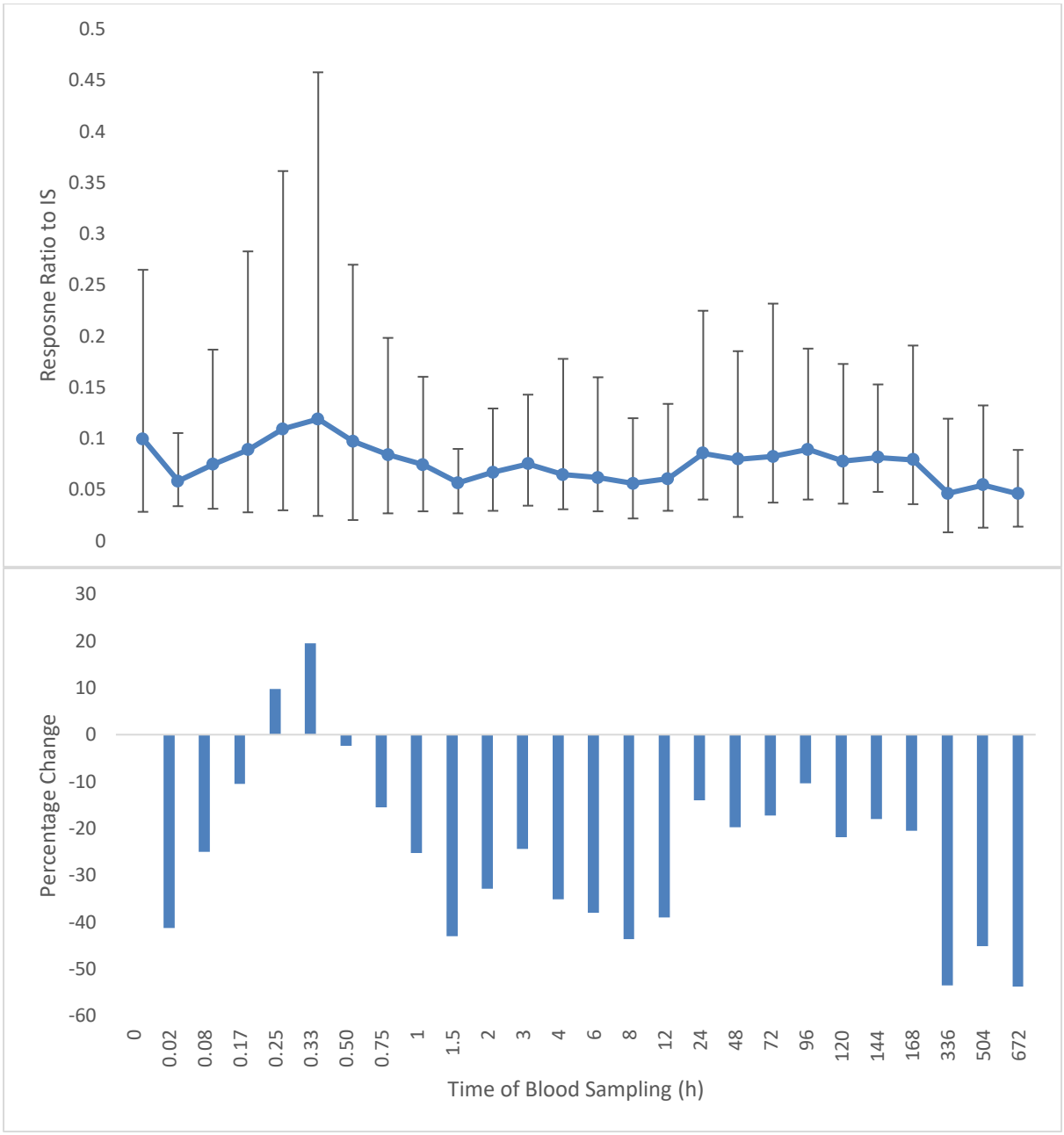

Figure A36: 15-HEDE profile following a TA administration. Top panel showing integrated peak area with vertical bars representing the range, bottom panel showing the average percentage change (n=8)

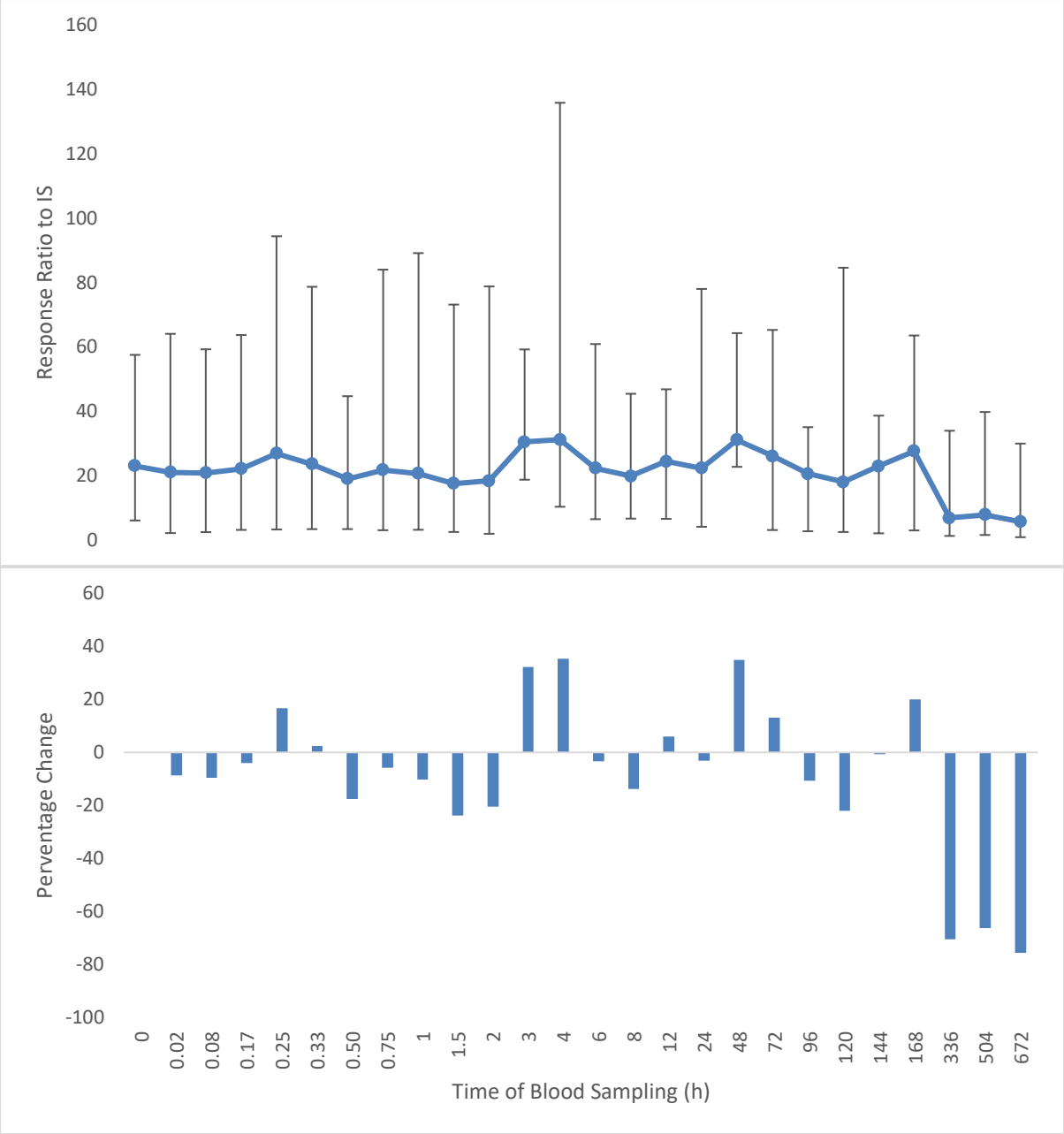

Figure A37: 9-HOTrE profile following a TA administration. Top panel showing integrated peak area with vertical bars representing the range, bottom panel showing average percentage change (n=8).

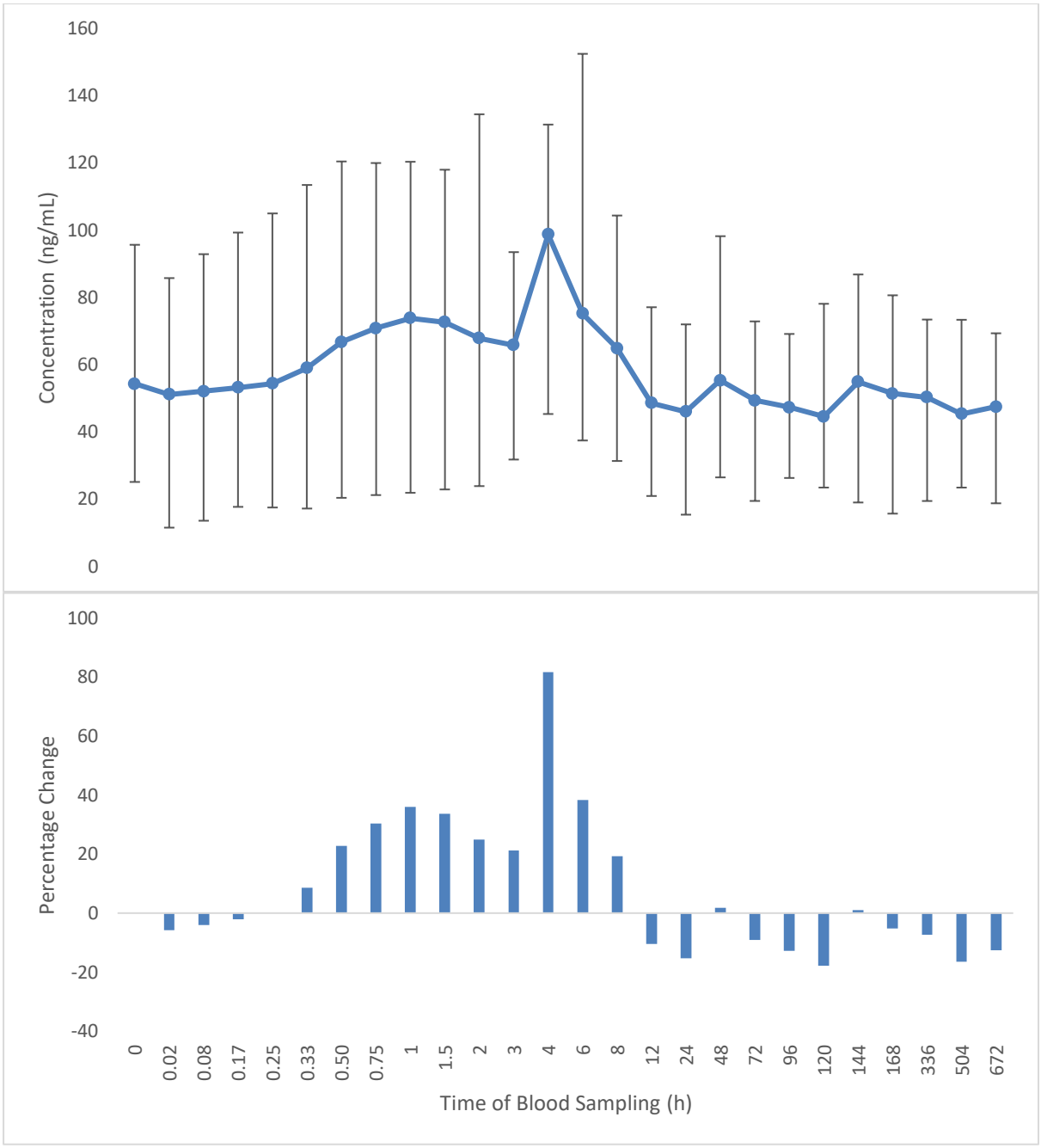

Figure A38: HC profile following a TA administration. Top panel showing estimated concentration with vertical bars representing the range, bottom panel showing average percentage change (n=8).

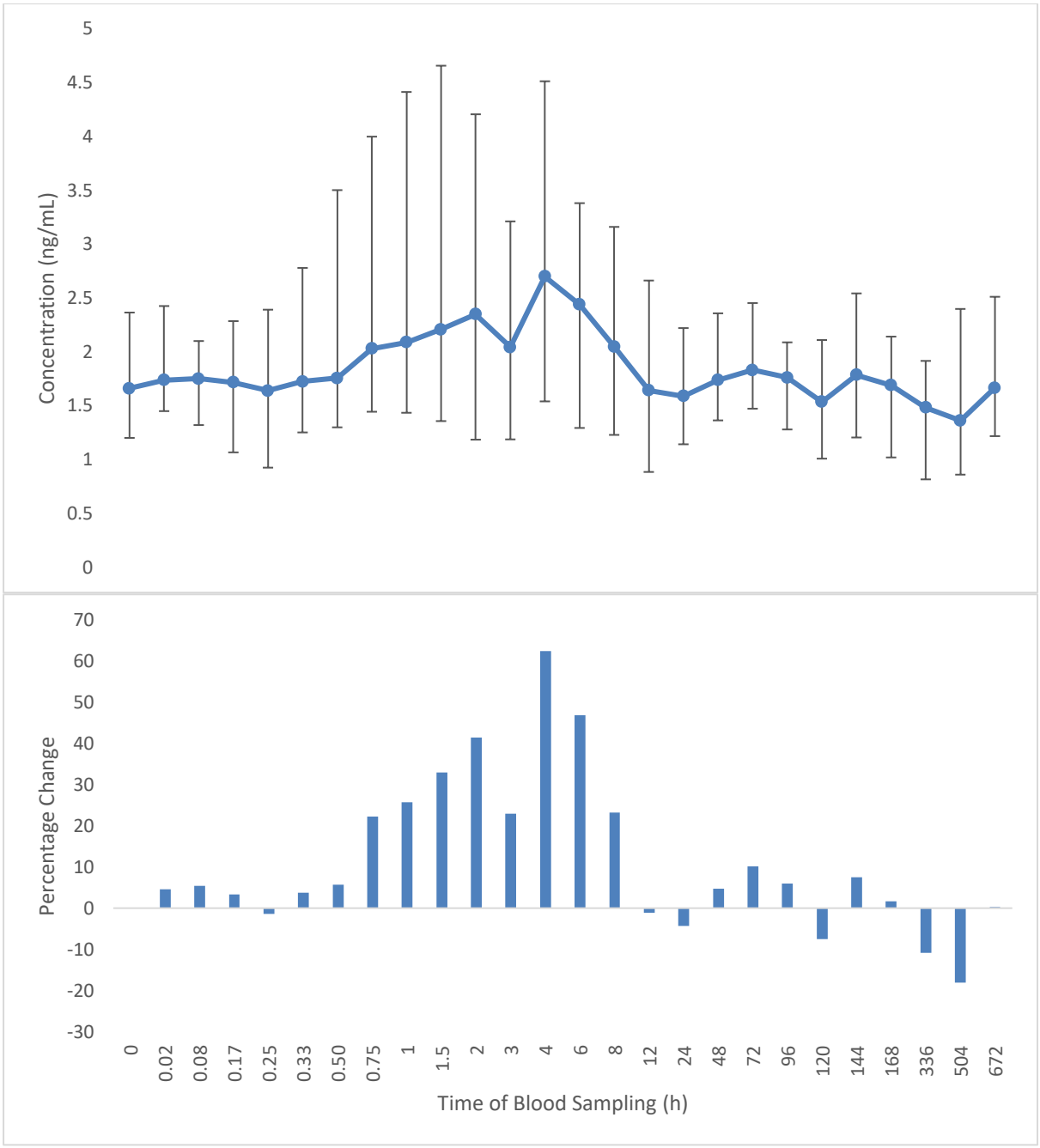

Figure A39: C profile following a TA administration. Top panel showing concentration with vertical bars representing the range, bottom panel showing average percentage change (n=8).

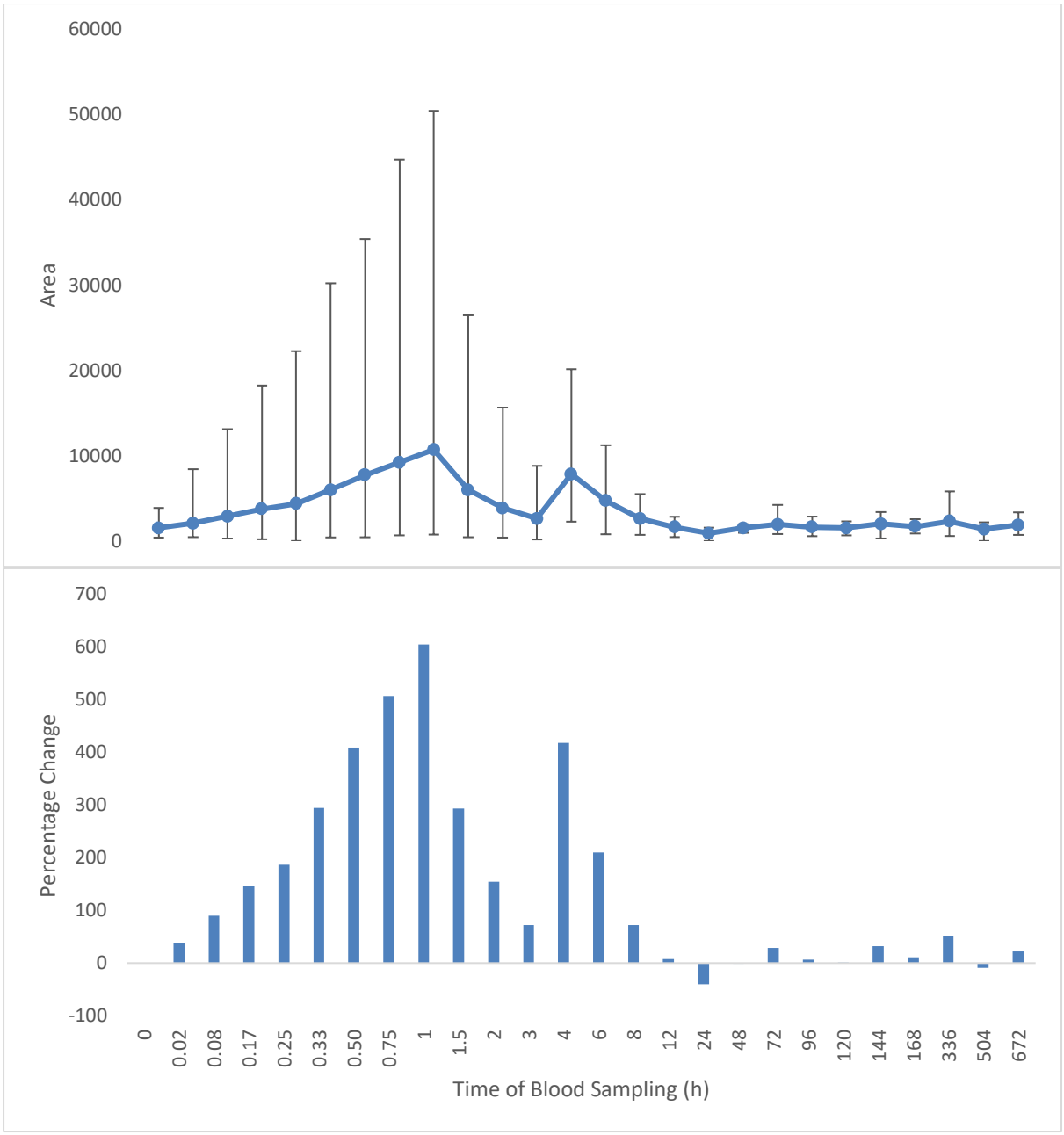

Figure A40: 11-deoxycortisol profile following a TA administration. Top panel showing peak area with vertical bars representing the range, bottom showing average percentage change (n=8).

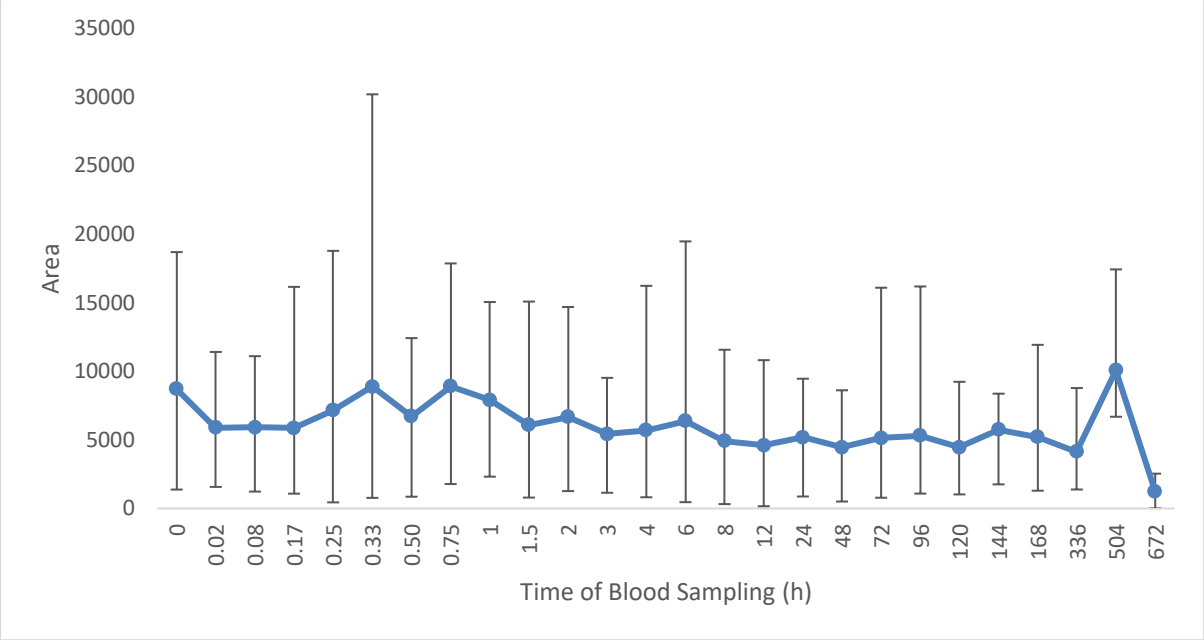

Figure A41: 10-HDHA profile following a TA administration using peak area. Vertical bars represent range.

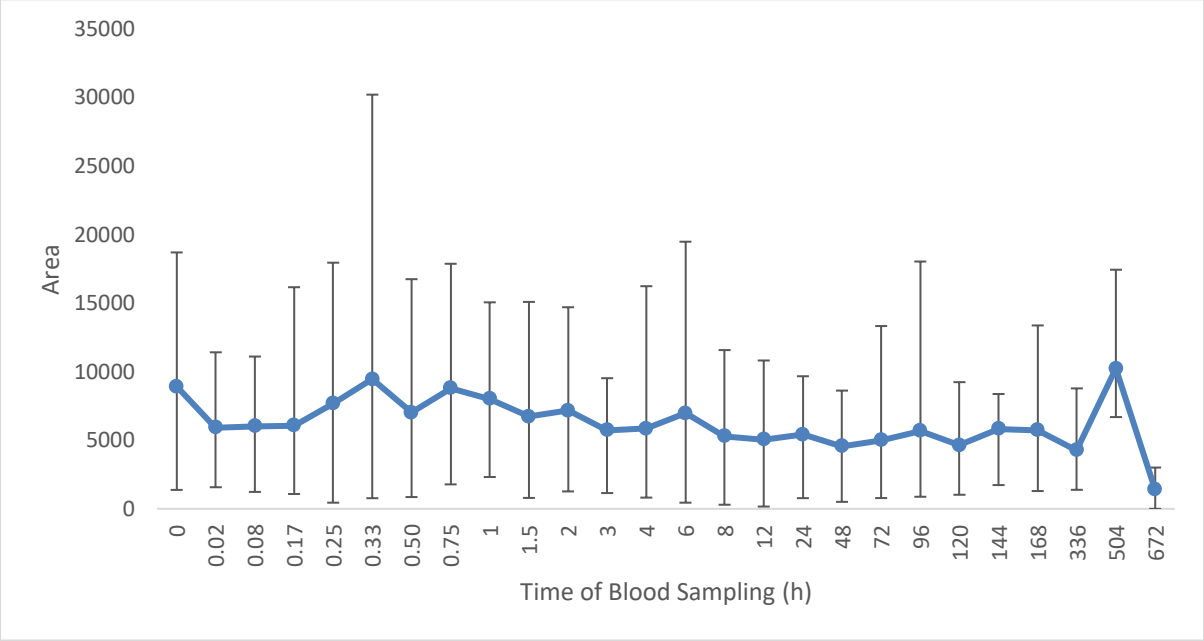

Figure A42: 14-HDHA profiling following a TA administration using peak area. Vertical bars represent range.

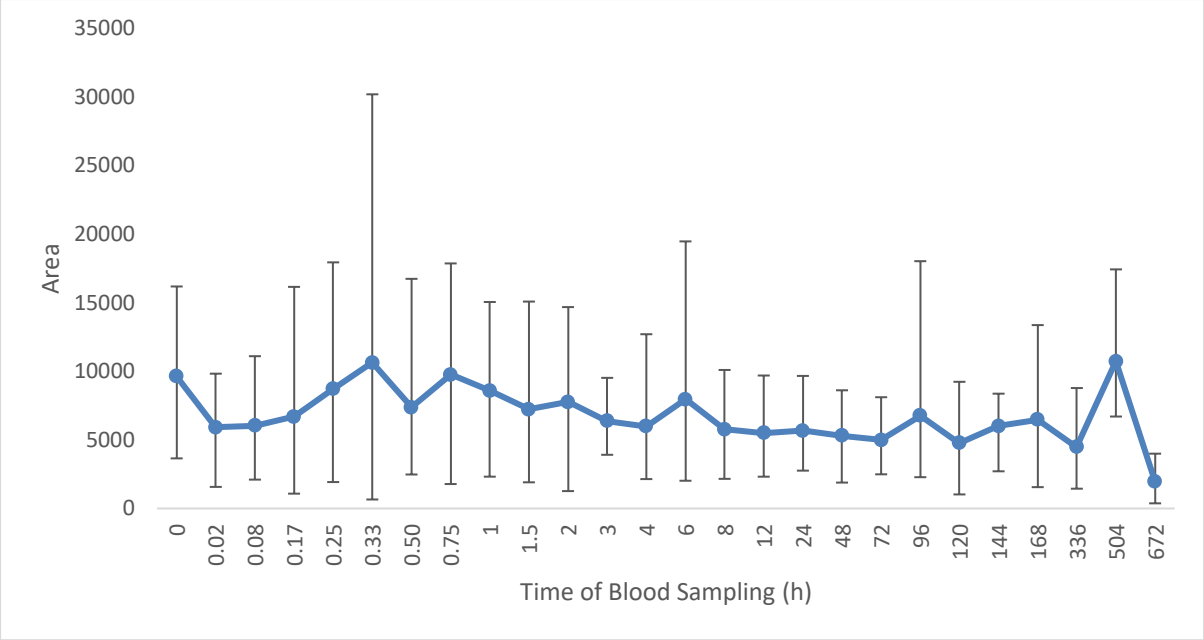

Figure A43: 7-HDHA profiling following a TA administration using peak area. Vertical bars represent range.

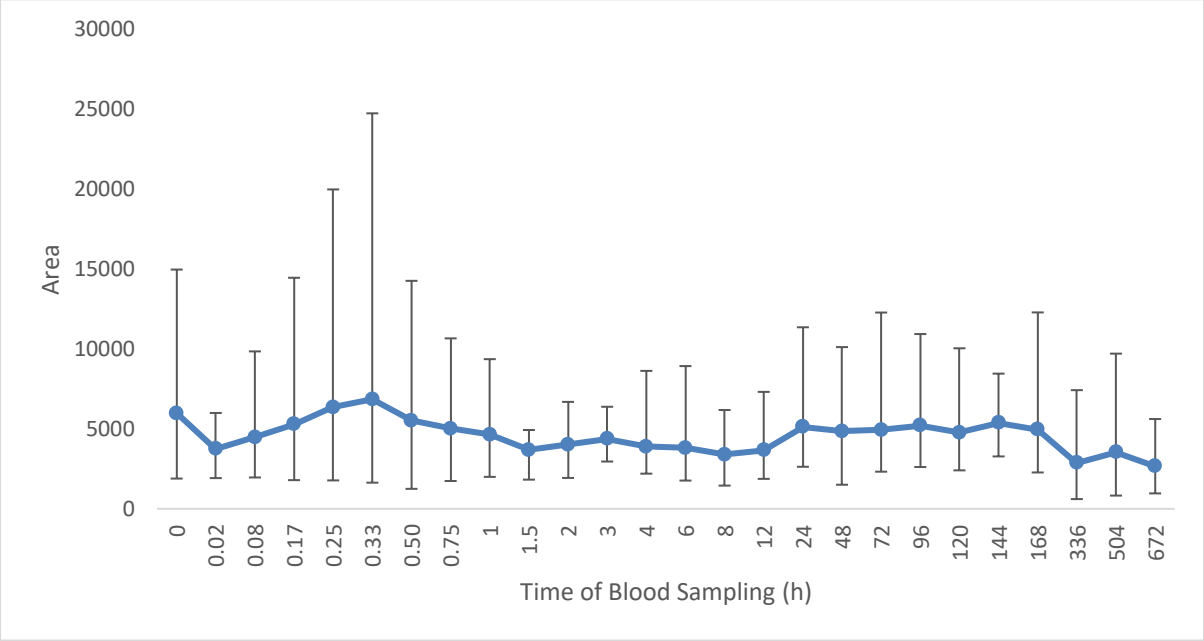

Figure A44: 11-HEDE profiling following a TA administration using peak area. Vertical bars represent range.

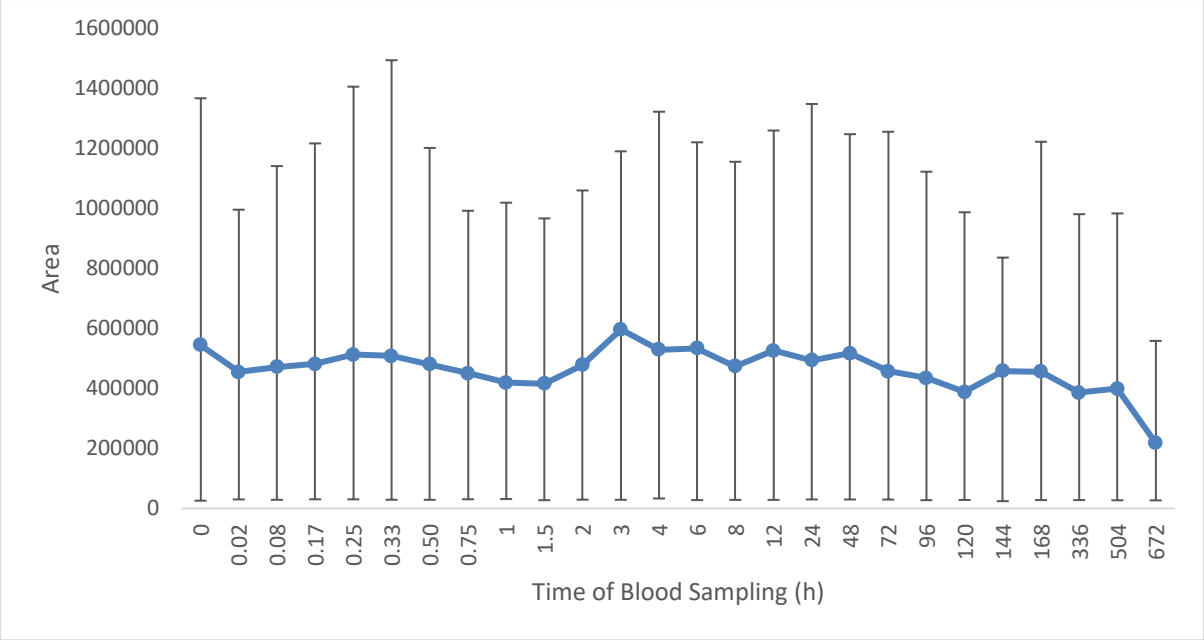

Figure A45: 13-HODE profiling following a TA administration using peak area. Vertical bars represent range.

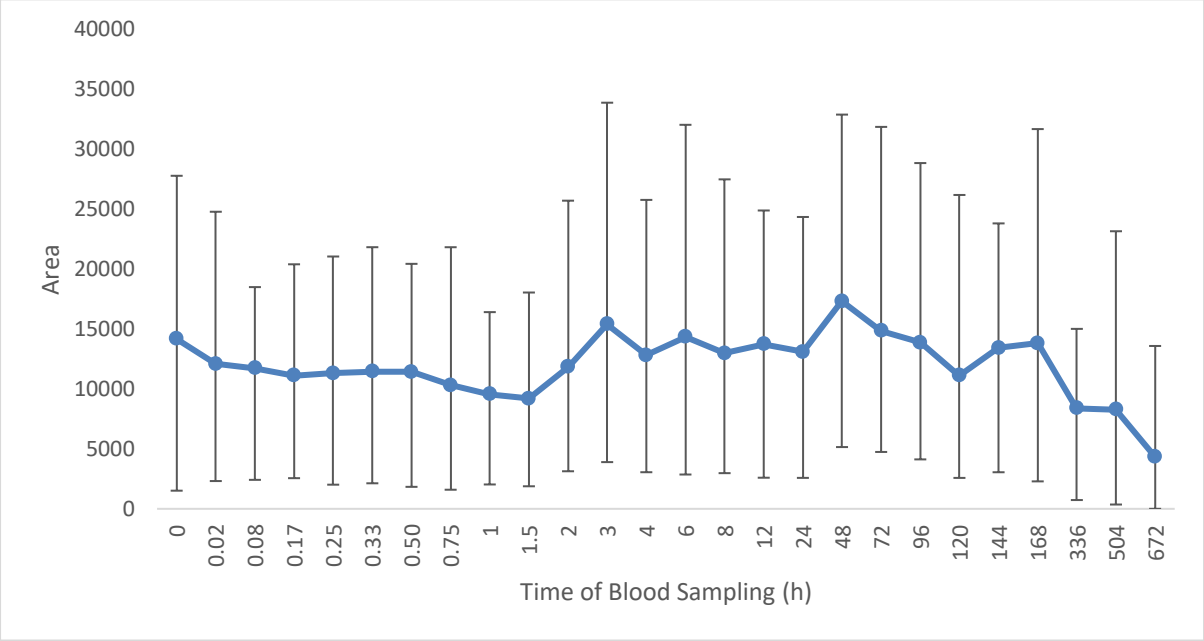

Figure A46: 4-HDHA profiling following a TA administration using peak area. Vertical bars represent range.

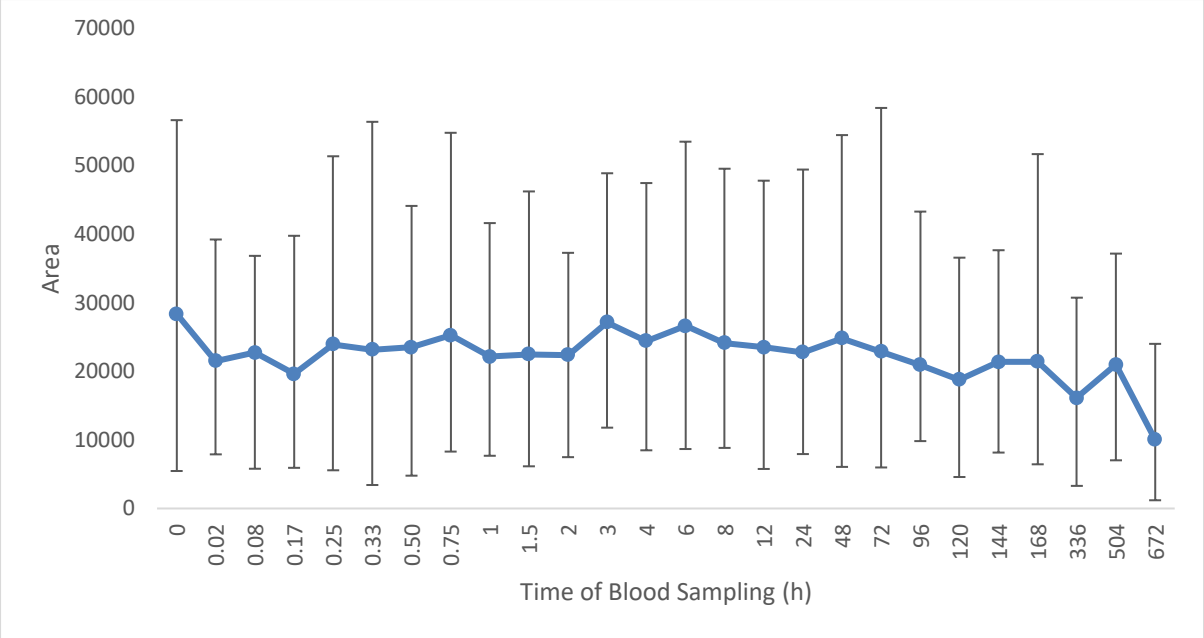

Figure A47: 9-HEPE profiling following a TA administration using peak area. Vertical bars represent range.

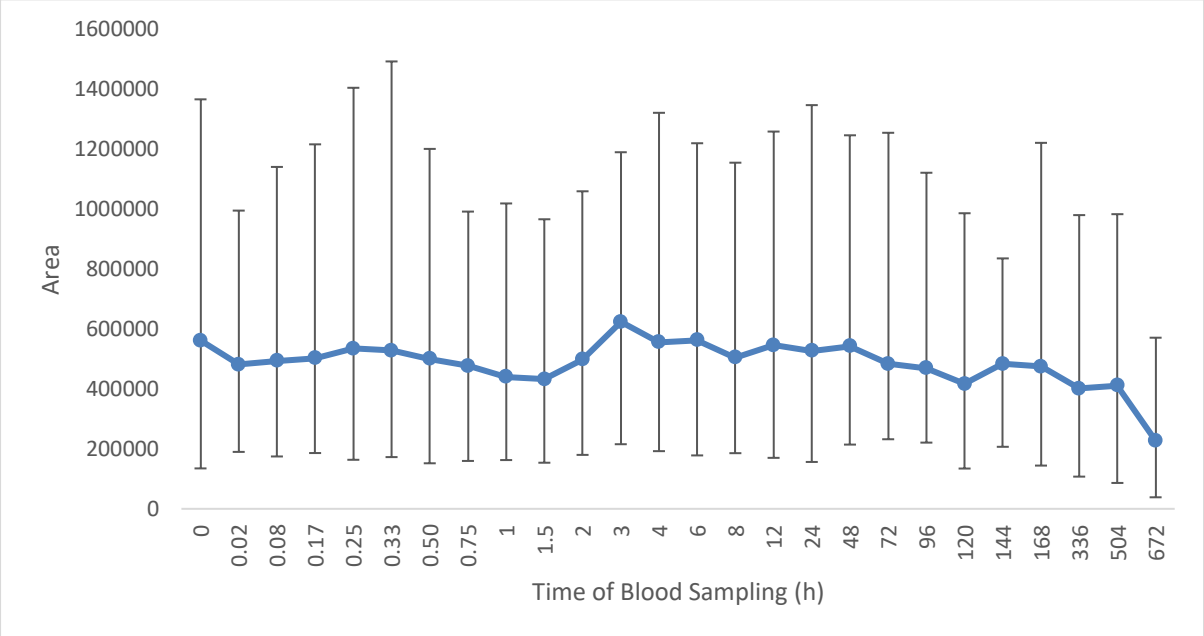

Figure A48: 9-HODE profiling following a TA administration using peak area. Vertical bars represent range.

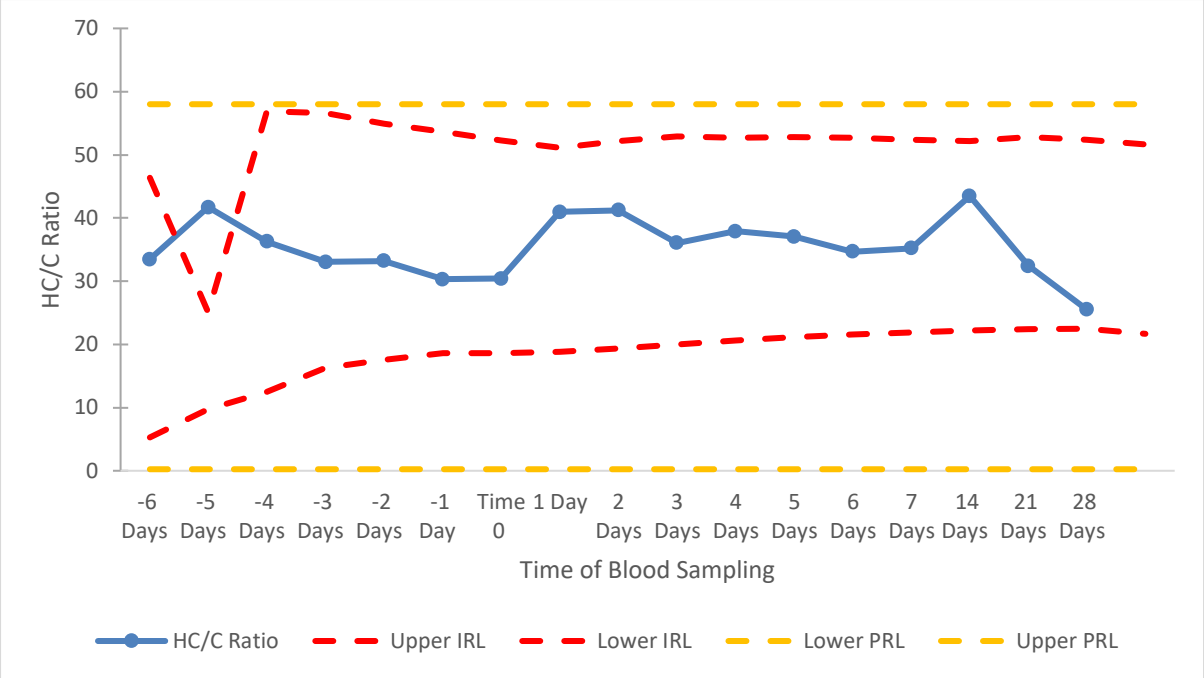

Figure A49: Intra-individual profile for the TA administration for Gelding A using the HC/C ratio (Legend: blue = HC/C ratio, red = individual upper and lower thresholds, yellow = proposed population upper and lower thresholds).

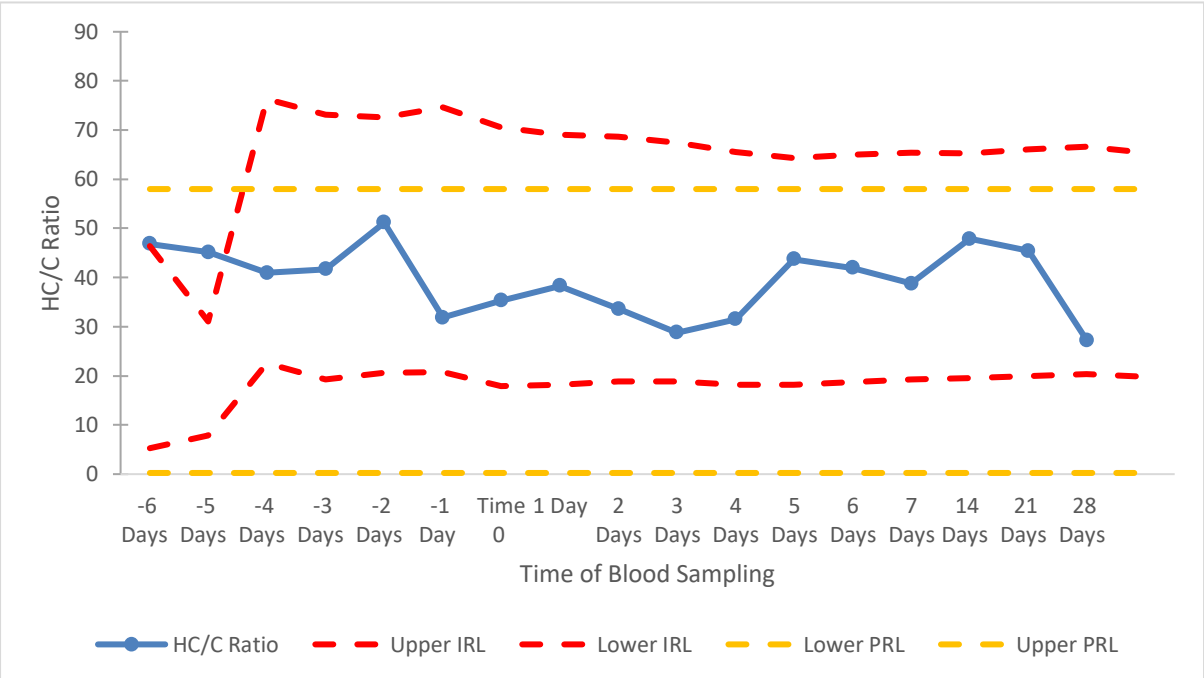

Figure A50: Intra-individual profile for the TA administration for Gelding B using the HC/C ratio (Legend: blue = HC/C ratio, red = individual upper and lower thresholds, yellow = proposed population upper and lower thresholds).

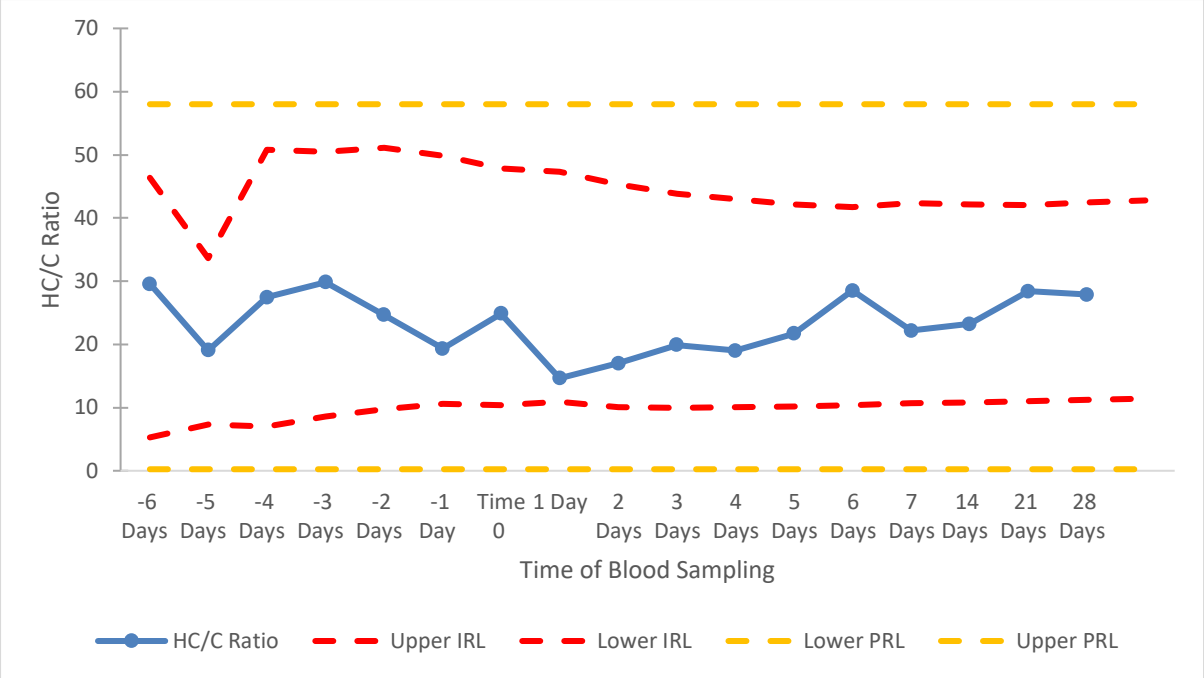

Figure A51: Intra-individual profile for the TA administration for Gelding C using the HC/C ratio (Legend: blue = HC/C ratio, red = individual upper and lower thresholds, yellow = proposed population upper and lower thresholds).

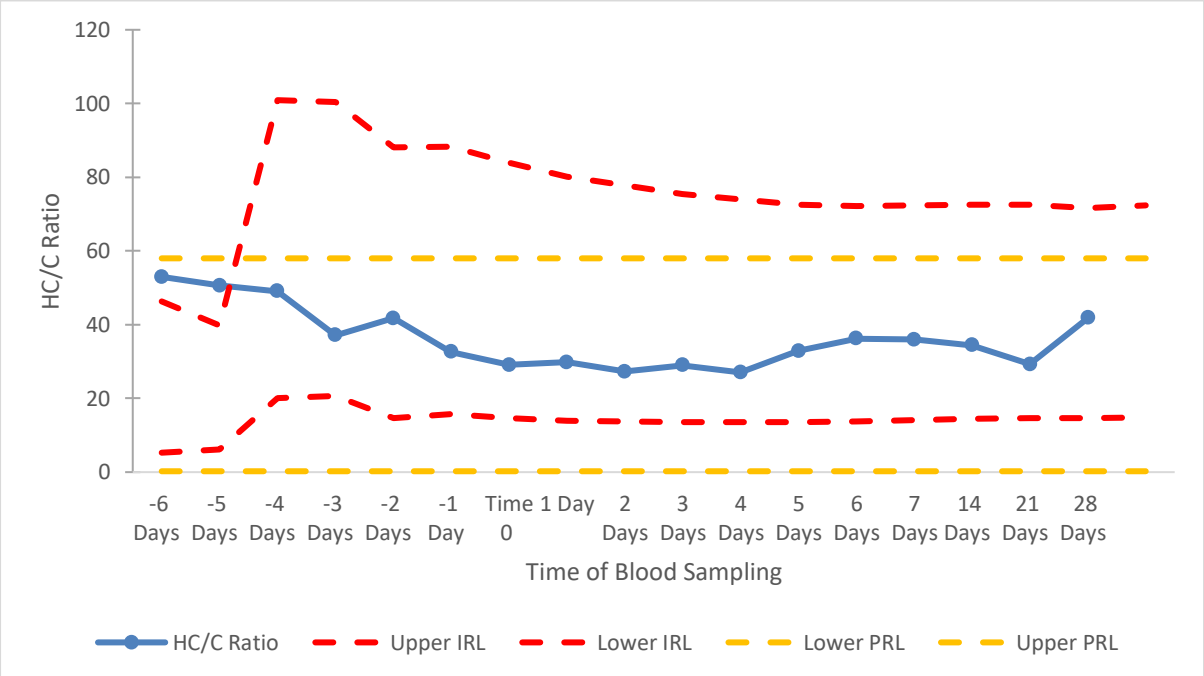

Figure A52: Intra-individual profile for the TA administration for Mare A using the HC/C ratio (Legend: blue = HC/C ratio, red = individual upper and lower thresholds, yellow = proposed population upper and lower thresholds).

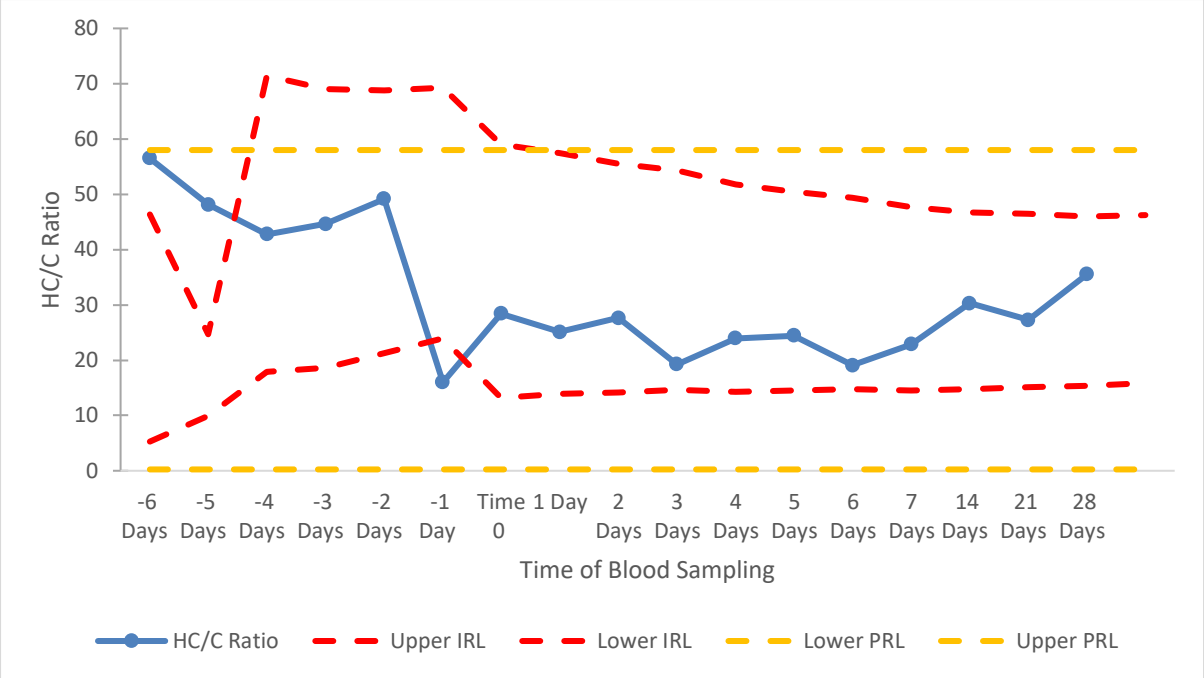

Figure A53: Intra-individual profile for the TA administration for Mare B using the HC/C ratio (Legend: blue = HC/C ratio, red = individual upper and lower thresholds, yellow = proposed population upper and lower thresholds).

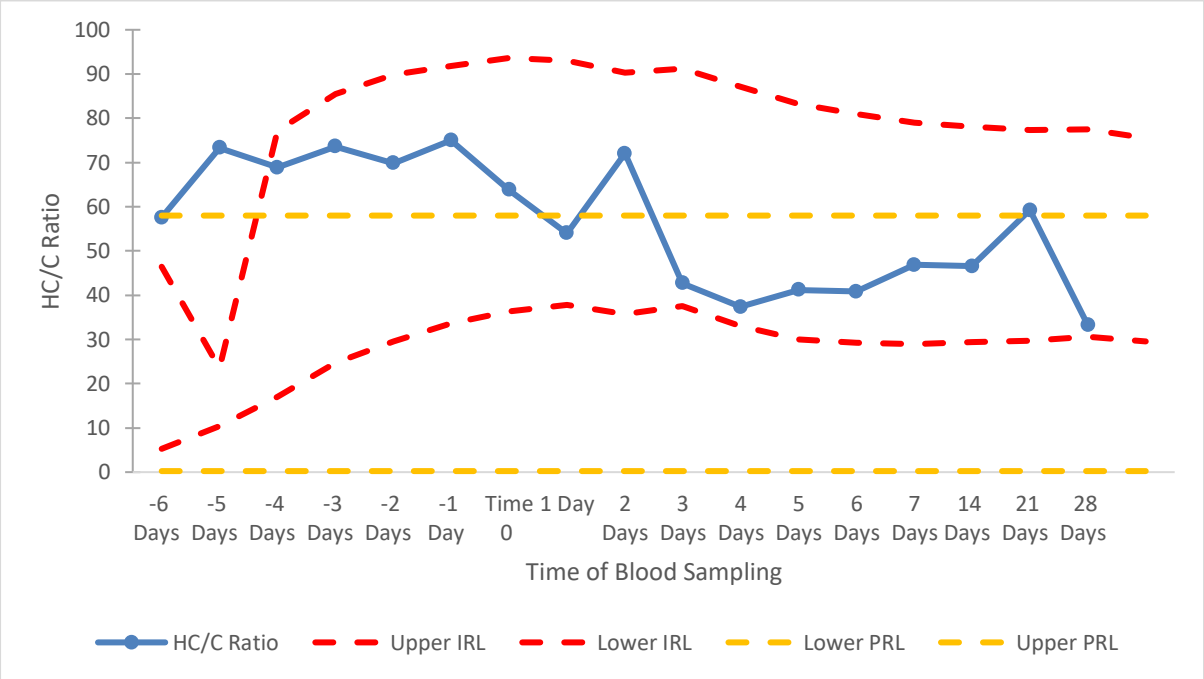

Figure A54: Intra-individual profile for the TA administration for Mare C using the HC/C ratio (Legend: blue = HC/C ratio, red = individual upper and lower thresholds, yellow = proposed population upper and lower thresholds).

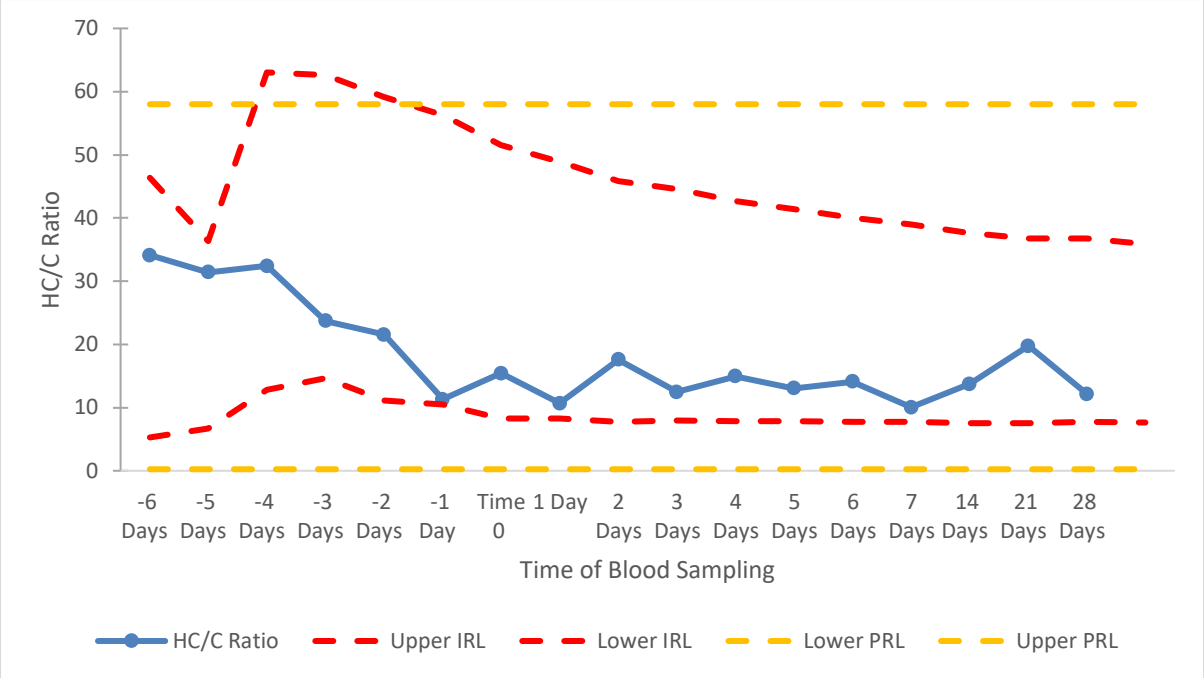

Figure A55: Intra-individual profile for the TA administration for Mare D using the HC/C ratio (Legend: blue = HC/C ratio, red = individual upper and lower thresholds, yellow = proposed population upper and lower thresholds).

Appendix 5: Research to Routine Translation

Instrument parameters for research to routine translation.

Table A22: MS Condition for LC-MS/MS 8060.

Conditions	**Setting**
Interface Temperature (°C)	400
DL Temperature (°C)	300
Heating Block Temperature (°C)	400
Drying Gas Flow (L/min)	15
Heating Gas Flow (L/min)	10
Nebulising Gas Flow (L/min)	10

Method validation preparation parameters

Linearity

Table A23: Plasma (2 mL) spike calibration preparation summary.

Spike Concentration (ng/mL)	Analyte Solution	Amount (µL)
0	-	-
1	Working 100 ng/mL	20
2	Working 100 ng/mL	40
5	Working 100 ng/mL	100
10	Working 100 ng/mL	200
50	Stock 2000 ng/mL	50

Sensitivity

Table A24: Plasma spike for LOD and LOQ preparation in 2 mL of equine plasma.

Spike Concentration (ng/mL)	Solution	Amount (µL)
0.01	Working 1 ng/mL	20
0.02	Working 1 ng/mL	40
0.025	Working 1 ng/mL	50
0.03	Working 1 ng/mL	60
0.04	Working 1 ng/mL	80

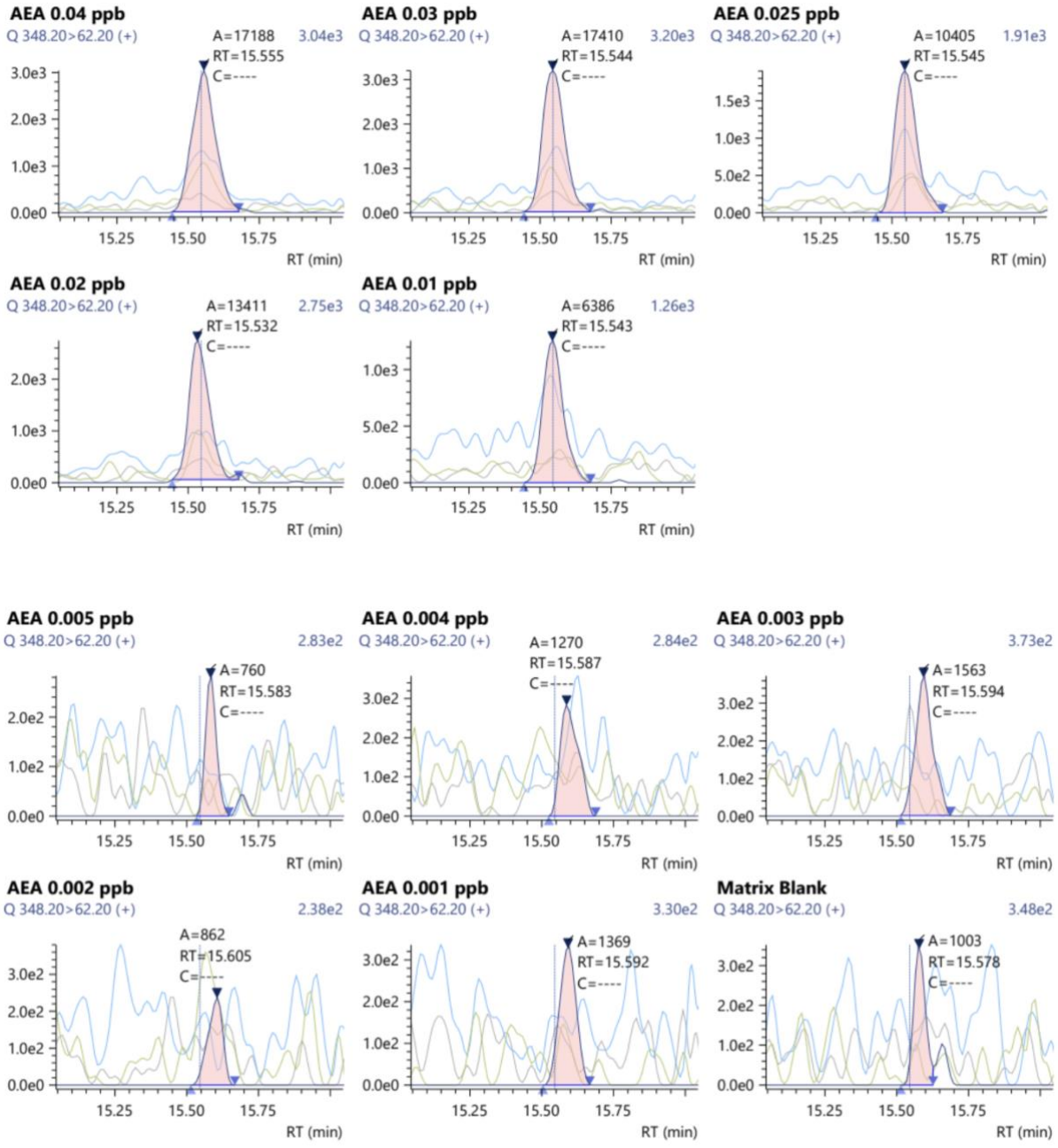

Figure A56: Chromatograms for AEA to determine LOD and LOQ for the 2 mL method.

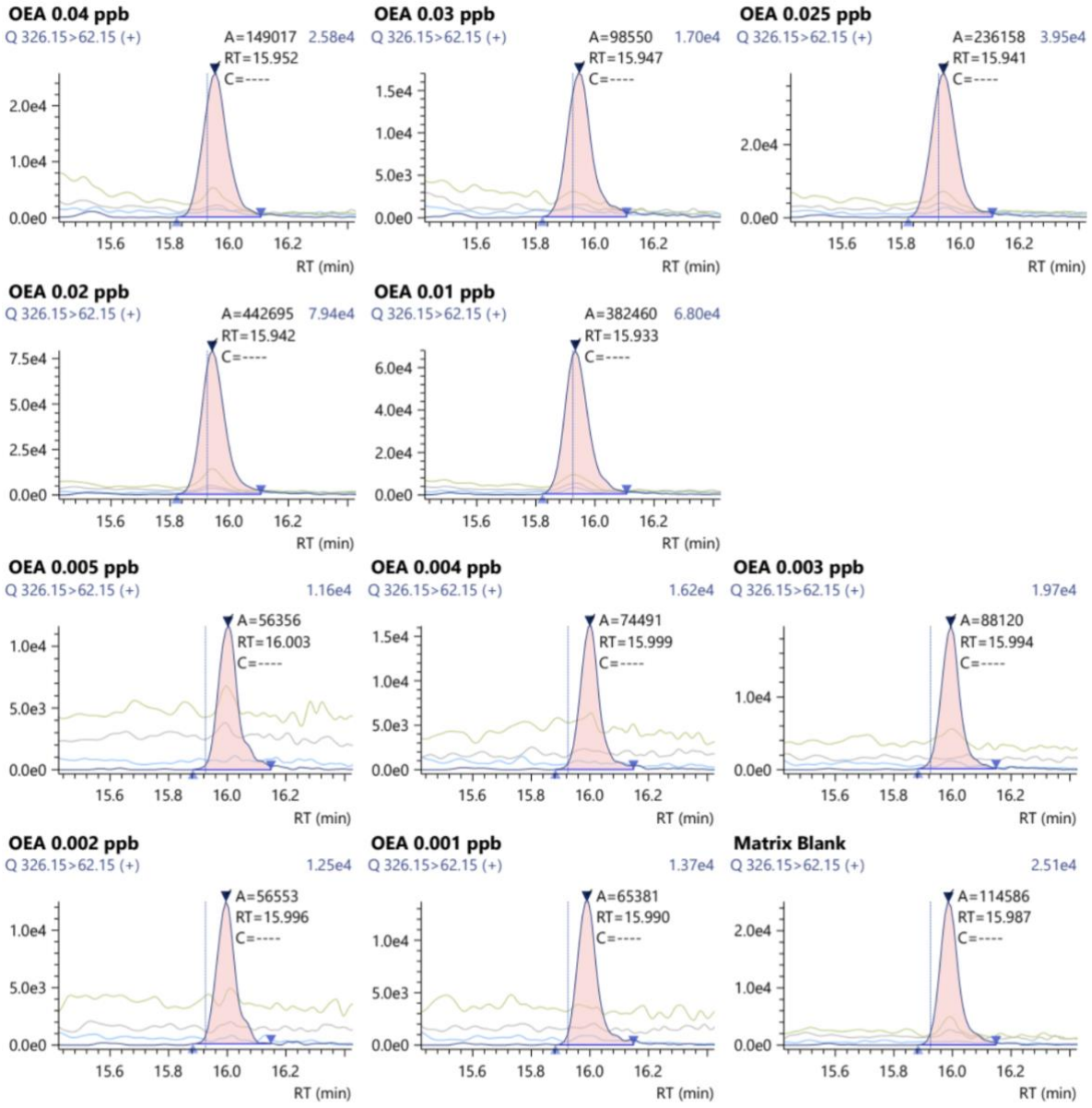

Figure A57: Chromatograms for OEA to determine LOD and LOQ for the 2 mL method.